VOLUME 41 [J. CELL. AND COMP. PHYSIOL.] NUMBER 3

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

JUNE 1953

PUBLISHED BIMONTHLY BY

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

WOODLAND AVENUE AND THIRTY-SIXTH STREET, PHILADELPHIA 4, PA.

Entered as second-class matter February 19, 1932, at the post office at Philadelphia, Pa., under Act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in section 1103, Act of October 3, 1917, authorized on July 2, 1918

Price, $7.50 per volume, Domestic; $8.00 per volume, Foreign

Publications of The Wistar Institute

JOURNAL OF MORPHOLOGY

Devoted to the publication of original research on animal morphology, including cytology, protozoology, and the embryology of vertebrates and invertebrates. Articles do not usually exceed 50 pages in length.

Issued bimonthly, 2 vols. annually: $20.000 Domestic, $21.00 Foreign, per year.

THE JOURNAL OF COMPARATIVE NEUROLOGY

Publishes the result of original investigations on the comparative anatomy and physiology of the nervous system.

Issued bimonthly, 2 vols. annually: $150.00 Domestic, $16.00 Foreign, per year.

THE AMERICAN JOURNAL OF ANATOMY

Publishes the results of comprehensive investigations in vertebrate anatomy — descriptive, analytical, experimental.

Issued bimonthly, 2 vols. annually: $15.00 Domestic, $16.00 Foreign, per year.

THE ANATOMICAL RECORD

Organ of the American Association of Anatomists and the American Society of Zoologists

For the prompt publication of concise original articles on vertebrate anatomy, preliminary reports; technical notes; critical notes of interest to anatomists and short reviews of noteworthy publications.

Issued monthly, 3 vols. annually: $22.50 Domestic, $24.00 Foreign, per year.

THE JOURNAL OF EXPERIMENTAL ZOOLOGY

Publishes papers embodying the results of original researches of an experimental or analytical nature in the field of zoology.

Issued 9 times a year, 3 vols. annually: $22.50 Domestic, $24.00 Foreign, per year.

AMERICAN JOURNAL OF PHYSICAL ANTHROPOLOGY

Organ of the American Association of Physical Anthropologists

Publishes original articles on comparative human morphology and physiology as well as on the history of this branch of science and the techniques used herein. In addition it gives comprehentive reviews of books and papers, an annual bibliography, and informal communications.

Issued quarterly, 1 vol. annually: $7.50 Domestic, $8.00 Foreign, per year.

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

Publishes papers which embody the results of original research of a quantitative or analytical nature in general and comparative physiology, including both their physical and chemical aspects.

Issued bimonthly, 2 vols. annually: $15.00 Domestic, $16.00 Foreign, per year.

THE JOURNAL OF NUTRITION

Organ of the American Institute of Nutrition

Publishes original researches in the field of nutrition and occasional reviews of literature on topics with which the journal is concerned.

Issued monthly, 3 vols. annually: $22.50 Domestic, $24.00 Foreign, per year.

THE AMERICAN ANATOMICAL MEMOIRS

Publishes original monographs based on experimental or descriptive investigations in the field of anatomy which are too extensive to appear in the current periodicals. Each number contains only one monograph. List of monographs already published, with prices, sent on application.

ADVANCE ABSTRACT CARD SERVICE

Every paper accepted for publication in one of the above periodicals is accompanied by the author's abstract. The abstract and the complete bibliography reference to the paper as it will eventually appear is printed on the face of a standard library catalogue card. This Advance Abstract Card Service is issued promptly, in advance of the journal containing the paper, and is offered in three styles.

Prices per year $2.00, $2.50 and $3.00, postpaid.

These publications enjoy the largest circulation of any similar journals published.

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

WOODLAND AVENUE AND THIRTY-SIXTH STREET, PHILADELPNIA 4, PA.

CELLULAR CHANGES ACCOMPANYING ACUTE AND SUB-ACUTE X-IRRADIATION DEATH OF THE HAMSTER [1]

II. THE ENDOCRINES

ROBERTS RUGH, BARNET LEVY [2] AND LOTTIE SAPADIN
Radiological Research Laboratory, Columbia University

FORTY-TWO FIGURES

INTRODUCTION

Radiation studies on the endocrine glands have been motivated largely by clinical interest in alleviating some pathological condition by destroying the gland with ionizing radiation and thereby impeding its function. There have been relatively few histopathological analyses of the changes attendant upon irradiation. On the whole, the endocrine organs have been considered radioresistant, following either direct exposure of the gland or total body radiation.

In discussing the effect of whole body x-irradiation upon the endocrine glands, one is immediately challenged by the realization that these organs (second possibly to the lymphocytic system) are quickest to reflect changes in the general body health of the organism. There may therefore be some confusion between systemic and direct effects, either of which might bring about specific histological changes. One has only to cite the effect of hypophysectomy on other endocrines to point up this problem. Nevertheless, in this study we have been interested in the composite whole (the hamster), even though we must of necessity treat the various organ systems

[1] This document is based on work performed under Contract AT-30-1-GEN-70 for the Atomic Energy Commission.

[2] From the Department of Dentistry.

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY, VOL. 41, NO. 3
JUNE 1953

of the body separately as though they were independent entities. In the final paper of this series we will attempt to evaluate the so called "organismic" factors.

While the literature suggests that the endocrines are radioresistant, we have found some x-irradiation effects on all of the endocrines studied. Since the effects on the various organs differ somewhat, and since we have also made a study of the acute and sub-acute radiation exposures, we propose to treat separately each of the major endocrine organ systems, namely: the thyroid, parathyroid, pituitary, adrenal, and pancreas.

The thyroid gland

Walters et al. ('32) claimed that the normal thyroid gland is quite radioresistant. The major effect was hyperplasia and, following an over dosage of x-radiation, a thickening of the capsule. Zimnitsky et al. ('36) irradiated male rabbits on successive days with 550 r and found that some of the thyroid glands showed hyperemia; some showed slight vacuolization of the cytoplasm; and others, following larger doses of x-radiation, showed a coarsening of the mitochondria. Warren ('43) reported that roentgen rays may stop, for a time, the production of colloid, rendering the follicle empty. He also stated that there may be vascular and connective tissue changes. It is of interest to note that Bloom ('48) and his co-workers on the Manhattan project did not include the endocrine glands in their over-all study of histopathological effects of radiation. Montag ('50) claimed that following 1500 r there were degenerative changes in the nervous connections with the thyroid and in the nuclei of the follicular cells. Most recently, Hursh et al. ('51) directed the x-ray beam to the thyroid glands alone, giving doses of 3,000 to 6,000 r. They found an increase in the I^{131} uptake in consequence, but they could identify no morphological changes in the gland. They regarded the hyperactivity of the gland as a reflection of the systemic response to radiation. They stated: "The level of local roentgen irradiation necessary to produce destruction

of thyroid tissue is greater than the highest dose which can be administered to a rat without causing its death."

The thyroid gland of control hamsters

The thyroid glands of the hamsters used as controls are normally active, and have epithelium that is uniformly cuboidal with the exception of a few follicles around the periphery of the organ in which the epithelium is flat (pl. 1, fig. 1). In these peripheral follicles the colloid is rather opaque and non-vacuolated, indicating inactivity. The vast majority of the follicles contain pale or thin colloid, with abundant evidence of secretory activity. The interfollicular stroma of these hamster thyroids appears to be quite vascular, but is definitely not hyperemic (pl. 1, fig. 4).

Thyroid of hamsters killed under the x-ray beam

The most apparent change in these thyroids is hyperemia and hemorrhage. The thyroid vessels are all engorged with blood (pl. 1, fig. 2), and there are numerous red blood cells scattered throughout the interfollicular stroma (pl. 1, fig. 5). Some blood cells may even be found embedded within otherwise normal colloid (pl. 1, fig. 7).

The colloid in the follicles of these hamsters is quite like that of the controls (pl. 1, compare figs. 1, 4 and 2, 5), with the more central follicles giving evidence of colloid resorption. The peripheral follicles seem to have retained their more opaque colloid. This condition is one which suggests that the resorptive activity of these thyroids has been stimulated.

The follicular epithelium is variable in height. In some areas it appears to be normal and secreting colloid while in other regions there appears to be cytoplasmic, but not nuclear, destruction. In general the follicular epithelium is more active than that of the controls (pl. 1, fig. 5). Some of the epithelium is cuboidal while the more peripheral follicles have flat epithelium. Pyknotic nuclei are present but are so rare that they are difficult to find. There is no evidence of karyorrhexis.

Thyroids of hamsters killed one week after an LD/100 dose of 1500 r

There is no evidence of hyperemia in these thyroids (pl. 1, fig. 3), but the presence of red blood cells in some of the colloid masses within the follicles indicates some hemorrhage (pl. 1, fig. 8).

Most of the colloid of these hamster thyroids is uniformly opaque, with no evidence of resorptive activity. The colloid appears to have been accumulated and "jelled" (pl. 1, figs. 3, 6, 8).

The follicular epithelium is quite uniformly cuboidal (pl. 1, fig. 6). Its smooth or flat inner surface indicates that there is no secretory activity going on. No pyknotic nuclei or mitoses can be found. There seems to be some cytoplasmic destruction.

The collagenous fibers which extend from the thyroid gland and into and around the parathyroid gland appear to have been coarsened and made fibrotic. The parathyroids are otherwise unaffected (pl. 1, fig. 8).

Summary on the thyroids

Hyperemia developed in the thyroids of hamsters killed under the x-ray beam but not in the thyroids of those hamsters which were given an LD/100 dose and killed a week later. Hemorrhage was seen in both instances, as evidenced by the presence of red blood cells within some colloid masses.

The colloid in the follicles of thyroids of hamsters killed under the beam was variable in opacity, but in general it appeared to have been resorbed to a slightly greater extent than in the controls. This suggested a slight stimulation to activity. This slight secretory hyperactivity was also noted in the follicular epithelium. It must be admitted that these conditions may reflect the generalized heightened metabolism of acute radiation death rather than a direct effect of radiations on the thyroid gland.

In contrast with the above, hamsters killed one week following an LD/100 dose of 1500 r had thyroid glands with uniformly opaque, concentrated and apparently "congealed" colloid which gave no evidence of being resorbed. The epithelium lining these follicles was of a uniform low cuboidal type, without evidence of secretory activity.

Nuclear damage was questionable, although a few pyknotic nuclei were found under both radiation conditions. There were only occasional suggestions of cytoplasmic damage in both instances. There was an apparent coarsening of the connective tissue (stroma) only in the thyroids of the delayed death series.

One might conclude that, aside from the general hyperemia, the thyroid and the parathyroid glands are quite radioresistant.

The parathyroids

Of all the endocrine organs studied the parathyroids exhibit the least histological effect following either acute or sub-acuate x-radiation exposures. Walters et al. ('31) described hyperemia of the parathyroids, and this was observed in our "death under the beam" series. These same investigators described an increase in the connective tissue of the parathyroids several months after dogs were exposed to x rays. We too found some increase in the connective tissue from the thyroid into and also around the parathyroid glands in those hamsters killed one week following the lethal dose of 1500 r. However, there was at no time any evidence of pyknosis, karyorrhexis, nuclear swelling, vacuolization of cytoplasm, or cell destruction of any kind. One must therefore consider the parathyroid gland to be the most resistant of all of the endocrine glands studied.

The pituitary gland

Direct x-radiation of the pituitary gland has been used in the treatment of certain functional disorders in the female (Suzuki, '31; Lacassagne and Nyka, '34, '35; Kotz and Parker,

'40). Denniston ('41, '42) found a differential susceptibility to x rays of the anterior lobe cells in the ground squirrel. The basophils seemed to be unaffected. The acidophils, with increasing doses of x-radiation, were decreased in number and the cytoplasm became cloudy and shrank while the nucleoli were enlarged. The blood sinuses became sclerotic and progressively engorged with erythrocytes. More recently Dougherty and White ('46) used radiation to study the pituitary-adrenal cortical control of lymphocytes. Freed et al. ('48) found that low exposures to x-radiation caused a slight enlargement of rat pituitaries with only transient effects on the normal pituitary function.

The effects of radiation on the pituitary gland of hamsters

When hamsters are killed under the x-ray beam there is hyperemia of the pars anterior and pars nervosa, but not of the pars intermedia (pl. 2, figs. 3, 4, 5). This may be due to the low vascular supply of the pars intermedia. The capillaries of the pars anterior appear to be more congested than those of the pars nervosa (pl. 2, figs. 4 and 5). Occasional extra-vascular erythrocytes may be seen. Isolated pyknotic nuclei are found in both the pars anterior and the pars nervosa, but they are infrequent and there is no evidence of karyorrhexis. The major effect of death under the beam on the pituitary gland is on its circulatory system rather than its cellular constituents.

When hamsters are killed one week following an LD/100 exposure of 1500 r the anterior lobe and the pars nervosa have only a few isolated pyknotic nuclei and there is no evidence of karyorrhexis. Some of the anterior lobe cells are swollen and a few nuclei are enlarged in diameter by at least 20% over the largest of the control nuclei (pl. 2, fig. 6). There seems to be a statistical increase in the relative number of acidophilic cells of the pars anterior, as distinguished by the Masson stain. Neither hyperemia nor hemorrhage is evident in any part of the pituitary gland (pl. 2, figs. 6, 7, 8). The

collagenous fibers between the crypts of cells appear to be coarsened (pl. 2, fig. 7). The fibers of the pars nervosa are no longer continuous threads but are granular and fragmented, suggesting some destruction (pl. 2, fig. 8).

Summary on the pituitary

The pituitary gland proved to be radioresistant as analyzed by histological methods. The pars intermedia exhibited no effects, but this may have been due to its low blood supply. The pars anterior and pars nervosa of hamsters killed under the beam showed hyperemia with very slight hemorrhage but animals given the LD/100 dose and killed a week later did not show these effects. Pyknosis was found in both instances, but it was rare. There was no evidence of karyorrhexis. In the slow death series there was slight nuclear swelling of the pars anterior cells and an apparent breakdown of the pars nervosa fibers.

The pancreas

Menetrier et al. ('09) irradiated the pancreas of a diabetic patient and found at autopsy that there was no change in this gland. Lazarus-Barlow ('22) exposed the pancreas of frogs, rats, rabbits and cats to the gamma rays of radium, and found that the islands of Langerhans were unaffected but that heavy exposures damaged the acinous cells whose supporting collagen was swollen. The epithelial nuclei were vesiculated, and there was some desquamation. Case and Warthin ('24) at autospy found lymphocytic infiltration of the pancreas of a patient exposed to a heavy dose of x-radiation over the epigastrium. Fibrosis of the pancreas was reported by Fisher et al. ('26) and by Leven ('33). Tsuzucki ('26) found the pancreas radioresistant except to very high doses. Rosenbaum ('27) irradiated the pancreas of hens and was unable to stop mitosis of islet cells, although there was some enlargement of the islands. Capocaccia and Vallebona ('29) stated positively that islet epithelium was more resistant than acinar epithelium. Hirsch ('31) found that following even very light radia-

tion the zymogen granules of the acinous cells were disturbed. Terbruggen and Heinlein ('32) produced lethal hypoglycemia in the rabbit after heavy doses of radiation, with accompanying pyknosis, degeneration, and vacuolization of the acinous cells. The island cells remained normal or were slightly hypertrophied. Shields Warren ('42) stated that "the pancreas is resistant to radiation injury."

The pancreas of the normal control hamsters has distinct islets of Langerhans (pl. 3, fig. 1), each surrounded by a thin reticular membrane (pl. 3, fig. 4). Zymogen granules in the acinar cells are abundant (Jewell and Charipper, '51).

Pancreas of hamsters killed under the x-ray beam

In the pancreas of these hamsters the islets of Langerhans are still distinct under low power magnification (pl. 3, fig. 2) and their constituent cells are not dispersed (pl. 3, fig. 5). The single reticular layer which normally surrounds the islet cells is usually broken, or discontinuous. From a third to a half of the islet cells have pyknotic nuclei, while others may exhibit various stages of chromatin condensation. The capillaries supplying the islets are often dilated and engorged with red blood corpuscles (pl. 4, fig. 7). The connective tissue cell nuclei and the red blood cells within the islets do not appear to be affected. The islet nuclei which are not pyknotic or which do not show chromatin condensation have an average diameter somewhat less than that of the control average (compare figs. 4 and 5, pl. 3).

Pyknosis is rarely seen in the nuclei of the acinar cells, although there are occasionally groups of cells with pyknotic nuclei. A few scattered instances of karyorrhexis may be found (pl. 4, figs. 11–13). Cytoplasmic vacuoles are sometimes seen (pl. 4, figs. 8, 9). There appears to be a reduction in the number and the staining quality of the zymogen granules.

The most pronounced effect of acute radiation on the pancreas, aside from the pyknotic nuclei, is the over-all hyper-

emia. The major capillaries are engorged; there are some extra-vascular (hemorrhagic) inter-acinar red blood cells, and the blood vessels of some of the islets are dilated.

Pancreas of hamsters killed one week following an LD/100 of 1500 r

The islets of Langerhans under these conditions appear to be reduced in number, although counts in whole glands were not taken. Since the reticular capsule around the islets is largely disintegrated (pl. 3, fig. 6) and the islet cells have become intermingled with acinous cells, the islets as such are much more difficult to locate (pl. 3, fig. 3).

The islet nuclei are quite uniform in appearance, and have an average diameter slightly greater than that of the controls. Pyknotic nuclei are rarely seen, but islet cell cytoplasm appears to be mingled with that of adjacent cytoplasm so as to provide a uniformly granular background. An occasional mitotic figure may be seen, indicating that x-radiation to 1500 r does not stop mitosis.

The acinar nuclei vary in diameter between 10–20 μ while the controls range from 10–12 μ (pl. 4, fig. 16). There are more large acinar nuclei in these animals than in the controls. Occasionally groups of these cells may have pyknotic nuclei, and they show some evidence of karyorrhexis (pl. 4, figs. 14, 15). No cytoplasmic vacuoles are found. The zymogen granules of these cells are definitely reduced in number and in staining quality.

The Laidlow stain indicates that there is some coarsening of the encapsulating as well as the stromal connective tissue of the pancreas in those hamsters killed one week after the lethal dose of 1500 r. This, however, is a difficult factor to evaluate.

General summary of effect of x rays on the pancreas

The effect of acute radiation damage on the pancreas was to damage the islet cells particularly, and to induce hyper-

emia. Some of the acinar cells developed vacuoles. There was some dissolution of zymogen granules.

The effect of slow radiation death on the pancreas was damage to the reticular connective tissue which encapsulates the islets of Langerhans, allowing the islet cells to be dispersed outwardly among the acinar cells. There was also some indication of fibrotic changes. There was no significant nuclear damage except for a slight swelling. The acinar cells showed reduction in their zymogen granules and some pyknosis and karyorrhexis. However, a few mitotic figures were observed.

The pancreas is quite radioresistant, but the islet cells are differentially more sensitive to acute radiation exposure than are the acinar cells.

The adrenal gland

The literature relating to the effects of x-radiation on the adrenal gland contains contradictory findings. Changes, ranging from hyperemia and hemorrhage (Harvey, '03; von Decastello, '12; Strauss, '21; Engelstadt and Torgersen, '37) through various degrees of necrosis (Cottentot et al., '12; Grabfield and Squier, '21; Wislocki and Crowe, '21; Lacassagne and Samssanow, '23; Warren, '43; Rhoades, '48 and others) have been described. Tsuzucki ('26) found a reduction in the fat content of the adrenal cortex, hyperemia and degeneration as well as atrophy of the medullary cord cells in animals exposed to high doses of irradiation. This is in direct contrast to the reports of the majority of other workers who have described the cortical cells as the more sensitive. Fibrosis of the adrenal gland following x-radiation has also been described. Recently Knowlton and Hempelmann ('49) studied the mitotic indices of the zona glomerulosa and zona fasciculata following various doses of x-radiation to the mouse adrenals, and found fluctuating response for the first three days followed by a slow but continuous decline.

With the exception of Tsuzucki's work, the conclusions may be drawn that the adrenal gland medulla is quite radioresist-

ant. The zona reticularis and zona glomerulosa are more sensitive than is the zona fasciculata; fat reduction, pyknosis, and cytoplasmic vacuolization are seen as signs of cell degeneration and fibrosis occurs over a long period following high exposure to x-radiation.

Adrenal gland of hamsters killed under the x-ray beam

There is slight hyperemia of the cortical layer, most apparent in the zona glomerulosa and the zona fasciculata in the form of dilated and engorged capillaries supplying the medulla.

Pyknotic nuclei in the cortex are very difficult to find. An occasional one may be located in the zona glomerulosa and a few more in the zona reticularis, but there are none in the zona fasciculata. Among the fasciculata cells, however, a few cytoplasmic vacuoles may be found. The average diameter of the fasciculata and reticulata cell nuclei is slightly greater than that of the control nuclei (pl. 5, fig. 4).

The medullary cords are distinct, not only because of the hyperemia and dilatation of their capillaries, which are engorged with red blood corpuscles, but also because the medullary region as a whole appears to be swollen (pl. 5, fig. 2). Some of the capillaries appear to be free of blood corpuscles, but do contain a fluid which is probably plasma. There is some evidence of hemorrhage within the medulla as well as a lymphocytic infiltration. There is extensive cytoplasmic destruction of the medullary cells (pl. 5, figs. 8, 9), with a few pyknotic nuclei. Some karyorrhexis is evident, particularly in association with the chromaffin as well as the epithelial cells. The major effect, however, is a swelling of the medullary cell nuclei, which are oval and measure as much as 17 μ in their long axes and 7 μ in their short axes (pl. 5, fig. 5), as compared with the nuclei of the controls, which are spherical and measure 6 to 7 μ in diameter. Nuclear swelling to this extent does not involve many cells, but most of the medullary

cell nuclei exhibit some swelling (i.e., they are 10–20% larger than the controls in diameter).

The adrenal capsule is intact and apparently unchanged.

Adrenal gland of hamsters killed one week after an LD/100 dose of 1500 r

Hyperemia of these adrenals is largely limited to the medulla, with dilatation of the blood vessels but without evidence of hemorrhage (pl. 5, fig. 3).

Pyknotic nuclei are virtually absent in the cortex (pl. 5, fig. 6), although occasionally a small group of such nuclei are found within the zona reticularis only, close to the medulla (pl. 5, fig. 7). Some cortical nuclei are slightly swollen, and the cytoplasm of some of these cells is vacuolated (pl. 5, fig. 6). The large vacuolar-like spaces among the zona fasciculata cells are much more abundant than in the control adrenals.

The medullary cells do not show the cytoplasmic damage found in the previous group, although a few scattered cells appear to be glandular, vacuolated, and in the process of disintegration (pl. 5, figs. 10, 11). Many of the nuclei of these cells are larger than those of the controls, but not to the extreme degree seen in the previous group. The control average is 6–7 μ and some of these nuclei measure up to 10 μ in diameter. Pyknosis is almost entirely absent. There is some pale basophilic staining material between the medullary cords, which may be edema fluid. The blood capillaries contain some fine plasma-like precipitate.

The capsule of these adrenals is definitely thickened. While the capsule around the control adrenals may vary in thickness from 7 to 17 μ, in these x-radiated hamsters the adrenal capsule varies from 9 to 22 μ in thickness.

Summary: adrenal gland

The adrenal gland is so sensitive to body stresses that it is difficult to isolate the changes attributable exclusively to the ionizing radiations. On the whole, the gland appears to be radioresistant. The degree of hyperemia is low; there are only

occasional pyknotic nuclei, and such damage as is manifest is related particularly to the cytoplasm of the medullary cord cells rather than to the cortex, and also to the over-all nuclear swelling. The slight thickening of the capsule in the slow death series is to be expected.

Summary and conclusions: the endocrines

1. The endocrine glands appear to be relatively radioresistant to acute x-radiation exposures. The current findings with hamsters differ in certain important details with some of the published conclusions. While parts of some of the glands are particularly sensitive, one might arrange the glands in the following order of increasing radiosensitivity: parathyroid, pituitary thyroid, adrenal, and pancreas.

2. All of the glands exhibit hyperemia in the "death under the beam series." This is most evident in the anterior pituitary and the thyroid, and least evident in the adrenal cortex and pars intermedia. Hemorrhage generally accompanies this hyperemia, particularly in the thyroid, the islets of Langerhans, and in the adrenal medulla.

3. Pyknosis is most abundant in the epithelial cells of the islets of Langerhans, but is also seen in the thyroid follicle epithelium, the anterior lobe of the pituitary, and to a slight extent in the adrenal medulla. Generally, where one finds pyknosis there is some accompanying karyorrhexis. Mitosis is abruptly stopped in all of these organs when the animal is killed under the beam.

4. Nuclear swelling is found throughout, both in "death under the beam" and the slower death following the lethal exposure. The swelling occurs to varying degrees and in different regions of the organs. It appears to be most pronounced in the adrenal and the pancreas.

5. Cytoplasmic damage precedes nuclear breakdown in most of the epithelial components such as the islets of Langerhans, the thyroid follicle, and the adrenal medulla.

6. The sub-acute exposure of 1500 r (animals sacrificed in one week) brought about the following effects: cessation of

secretory and resorptive activity of the thyroid follicular epithelium; reduction in the number and size of the zymogen granules of the acinar cells of the pancreas; reduction but not complete cessation of mitosis; some edema; overall swelling of nuclei, but not to any uniform degree even within the same part of the same organ; breakdown of connective tissue framework (reticular capsule around the islets of Langerhans); and increased fibrosis (thyroid and adrenal capsule). There was no hyperemia in these endocrine glands.

LITERATURE CITED

Bloom, W. 1948 Histopathology of irradiation. McGraw-Hill and Co.

Capocaccia, M., and A. Vallebona 1929 Alterazioni istologiche del negli animali trattati coi raggi roentgen. Arch. di biol., *6:* 13.

Case, J. T., and A. S. Warthin 1924 The occurrence of hepatic lesions in patients treated by intensive deep roentgen irradiation. Am. J. Roentgen. and Radium Therapy, *12:* 27.

Cottentot, P. H., A. Mulon and A. Zimmern 1912 Action des rayons x sur la corticale surrenale. Compt. rendu. Soc. de Biol., *73:* 717.

von Decastello, A. 1912 Ueber veranderungen au den nebennieren nach Roentgenbestrahlung. Wien. klin. Wchmschr. *25:* 759.

Denniston, R. 1941 Roentgen irradiation studies on pituitary and sex glands. Anat. Rec., *79:* 18.

———— 1942 The influence of roentgen ray treatments of the hypophysis on reproductive systems of the ground squirrel and rat. J. Exp. Zool., *91:* 237.

Desjardins, A. 1928 The effect of irradiation on the suprarenal gland. Am. J. Roentgen. and Radium Therapy, *19:* 453.

Dougherty, T. F., and A. White 1946 Pituitary-adrenal cortical control of lymphocyte structure and function as revealed by experimental x-radiation. Endocrinology, *39:* 370.

Engelstadt, R. B., and O. Torgerson 1937 Experimental investigations on the effects of roentgen rays on the suprarenal glands in rabbits. Acta radiologia, *18:* 671

Fisher, N. F., J. T. Groat and A. Bachem 1926 The effects of x-rays on the pancreas. Am. J. Physiol., *76:* 299.

Freed, J. H., E. J. Farris, D. P. Murphy and E. P. Pendergrass 1948 The effect of low dosage roentgen radiation on the gonadotrophic function of the hypophysis of the mature and the immature female albino rat. J. Clin. Endocrin., *8:* 461.

Grabfield, G. P., and T. L. H. Squier 1921 A note on the effect of irradiation of the suprarenal region in rabbits with roentgen rays. Arch. Int. Med., *27:* 168.

Harvey, W. G. 1908 On the pathological effects of roentgen rays on animal tissues. J. Path. Bacteriol., *12:* 549.

HIRSCH, G. C. 1931 Analyse der Restitution des Sekretmateriels im Pankreas mittels Rontgenstrahlen. Beobachtungen an Lebenden Zellen. Arch. f. Entwichlungsmech. der Org., *123:* 792.

HOLFELDER, H., AND H. PEIPER 1923 Die strahlenempfindlichkeit der nebennieren und wege zue verhütung von nebennierenschädigungen in der röntgentiefentherapie. Strahlentherapie, *15:* 1.

HURSCH, J. B., P. A. VAN VALKNEBERG AND J. B. MOHNEY 1951 Effect of roentgen radiation on thyroid function in rats. Radiology, *57:* 411.

KNOWLTON, H. P., AND L. H. HEMPELMANN 1949 The effect of x-rays on the mitotic activity of the adrenal gland. J. Cell. and Comp. Physiol., *33:* 73.

KOTZ, J., AND E. PARKER 1940 Treatment of functional disorders of the female by radiation of the pituitary gland. South. Med. Assoc. J., *33:* 832.

JEWELL, H. A., AND H. A. CHARIPPER 1951 The morphology of the pancreas of the golden hamster, cricetus amatus, with special reference to the histology and cytology of the islets of Langerhans. Anat. Rec., *111:* 401.

LACASSAGNE, A., AND W. NYKA 1934 Sur les processes histologiques de la destruction de l'hypophyse par le radon. Compt. rendu. Soc. de Biol., *117:* 956.

———— 1935 Modifications de l'appareil genital de la lapine secondairement a la destruction de l'hypophyse par la radon. Compt. rendu. Soc. de Biol., *119:* 979.

LACASSAGNE, A., AND N. SAMSSANOW 1923 De l'effet de la destruction totale ou partielle des capsules surrenales par le rayonnement caustique de foyers radioactifs. Comp. rendu. Soc. de Biol., *89:* 72.

LAZARUS-BARLOW, W. S. 1922 Studies of the effects of gamma rays from a large quantity of radium. Med. Res. Council, Spec. Rep. Ser. no. 62, London.

LEVEN, N. L. 1933 An experimental study; the effect of radium emanation on the pancreas of dogs. Am. J. Cancer, *18:* 899.

MARTIN, C. L., F. T. ROGERS AND N. F. FISHER 1924 The effect of roentgen rays on the adrenal gland. Am. J. Roentgen. and Radium Therapy, *12:* 466.

MENETRIER, P., A. TOURAINE AND R. MOLLET 1909 Action des rayons X chez les diabetiques. Bull. et mem. Soc. Med. d. Hop. de Paris, *28:* 658.

MONTAG, C. 1950 On the effect of methylthiouracil and x-rays on the thyroid gland. Strahlentherapie, *81:* 1.

RHOADES, R. P. 1948 The adrenal. Chap. 17 in Bloom's Histopathology of irradiation. McGraw-Hill and Co.

ROGERS, F. T., AND C. L. MARTIN 1930 The effects of x-rays on the adrenal gland. Am. J. Physiol., *93:* 219.

ROSENBAUM, W. 1927 Regeneration des pankreas nach Roentgenbestrahlung. Wien. klin. Wchnschr., *40:* 1319.

STRAUSS, O. 1921 Zum verhalten des blutdruchs nach Roentgenbestrahlung. Fortschr. a. d. Geb. d. Roentgenstrahlen, *28:* 467.

SUZUKI, S. 1931 The influence of the irradiation of the hypophysis upon the female genitals. Japan J. Obstet. and Gynec., *14:* 393.

TERBRUGGEN, A., AND H. HEINLEIN 1932 Hypoglykamie nach experimenteller Roentgenbestrahlung des pankreas. Kin. Wchnschr., *11:* 1139.

TSUZUKI, M. 1926 Experimental studies on the biological action of hard roentgen rays. Am. J. Physiol., *16:* 134.

WALTERS, O. M., B. J. ANSON AND A. C. IVY 1931 The effects of x-rays on the thyroid and parathyroid glands. Radiology, *16:* 52.

———— 1932 The prevention of hyperplasia of the thyroid in the opossum by x-rays. Radiology, *18:* 583.

WARREN, S. 1942 Effects of radiation on normal tissues. IV. Effects of radiation on the gastrointestinal tract, including the salivary glands, the liver and the pancreas. Arch. Path., *34:* 749.

———— 1943 Effects of radiation on normal tissues. XI. Effects on the endocrine glands. Arch. Path., *35:* 313.

WISLOCKI, G. B., AND S. J. CROWE 1922 Notes on the abdominal chromaffin body in dogs. Bull. Johns Hopkins Hosp., *33:* 377.

ZIMNITSKY, B. S., N. A. BASKINA AND A. P. DEVIRZ 1936 The effect of x-rays upon the fine structure of the parenchyma of the thyroid gland. Radiology, *27:* 175.

PLATES

PLATE 1

EXPLANATION OF FIGURES

The thyroid of hamsters

1–3 Low power magnification of thyroid sections.

1 — Control hamster.
2 — Hamster killed under the x-ray beam, showing hyperemia and some evidence of hyperactivity.
3 — Hamster killed one week after receiving and LD/100 of 1500 r, showing hypofunction with accumulation of opaque colloid.

4 [1] Control hamster thyroid showing normally active follicular epithelium and resorbing colloid.

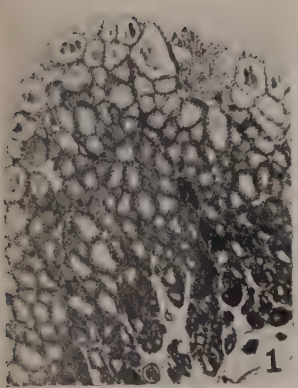
5 Thyroid of hamster killed under the x-ray beam showing slight hyperactivity but with some epithelial (cytoplasmic) destruction, inter-follicular occlusion of corpuscles, and occasional pyknotic nuclei.

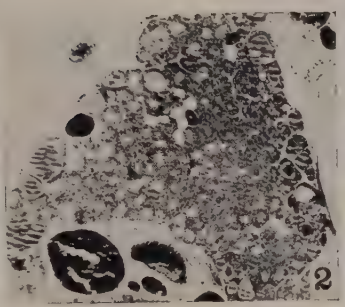
6 Thyroid of hamster killed one week after an LD/100 exposure showing inactive follicular epithelium and the accumulation of opaque colloid.

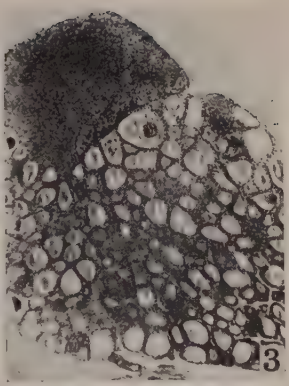
7 Same thyroid as figures 2 and 5 except showing invasion of follicular colloid by red blood corpuscles, giving evidence of hemorrhage.

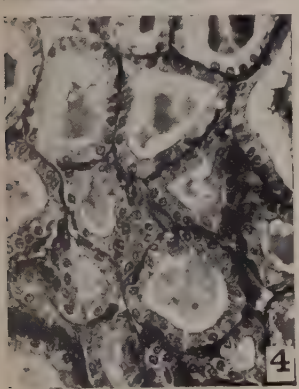
8 Same thyroid as figures 3 and 6 but showing red blood corpuscles within the follicular colloid and normal-appearing parathyroid cells (to upper right).

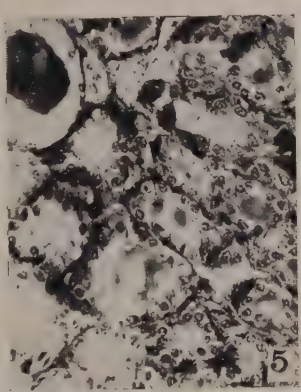
[1] Figures 4 to 8 inclusive are enlarged to the same degree.

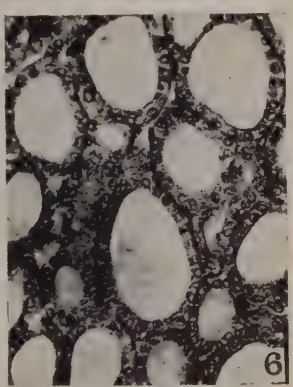

1

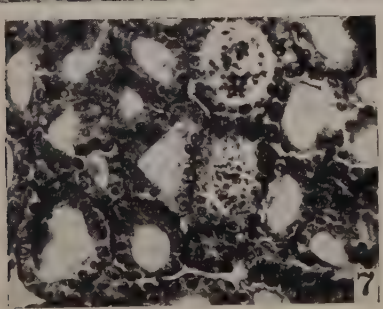

2


3


4


5


6


7

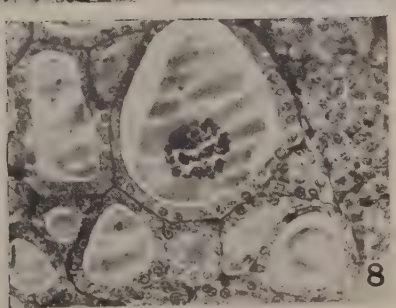

8

PLATE 2

EXPLANATION OF FIGURES

The pituitary gland of hamsters

1 Control pituitary enlarged to show pars anterior (above), interlobular space, pars intermedia (beneath space) and fibrous pars nervosa (lower region).

2 Enlarged section of anterior lobe showing normal acidophils, basophils, and chromophobes.

3 Low power magnification of entire pituitary gland of hamster killed under the x-ray beam showing hyperemia or engorgement of the blood capillaries of the anterior lobe particularly.

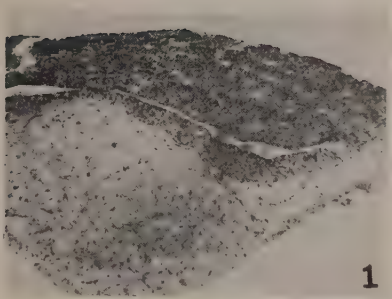
4 Enlarged section of anterior lobe of pituitary (same as fig. 3) showing extreme occlusion of blood capillaries. One pyknotic nucleus may be seen just above the center of the field.

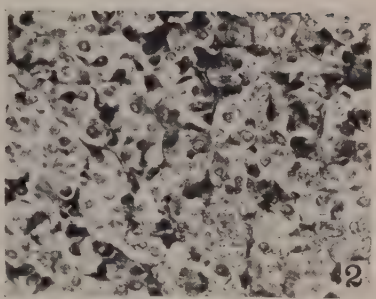
5 Pars nervosa of hamster killed under the beam showing hyperemia.

6 Pars anterior of hamster killed one week after an LD/100 dose of 1500 r showing some cytoplasmic destruction (compare with fig. 2) but no hyperemia. Note swelling of cells and nuclei (one large basophil toward center of field might be considered a giant cell).

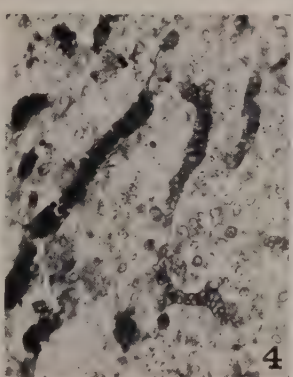
7 Apparent coarsening of inter-crypt connective tissue of the anterior lobe of hamster shown in figure 6.

8 Pars nervosa of hamster shown in figures 6 and 7 showing lack of hyperemia, but slight destruction (granulation) of fibers.

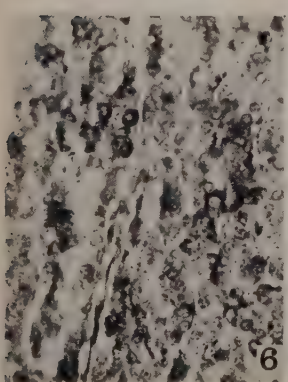

1

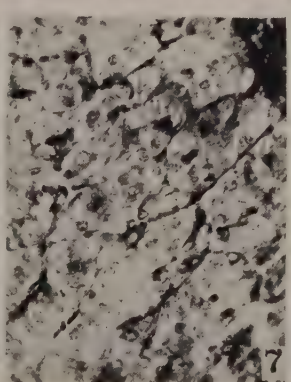

2


3


4


5


6


7

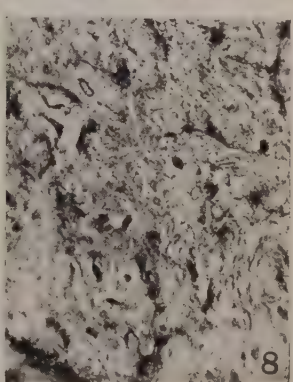

8

PLATE 3

EXPLANATION OF FIGURES

The pancreas of hamsters

1 Low power photograph of section of control hamster pancreas, showing clearly demarked islet of Langerhans and interlobular duct.

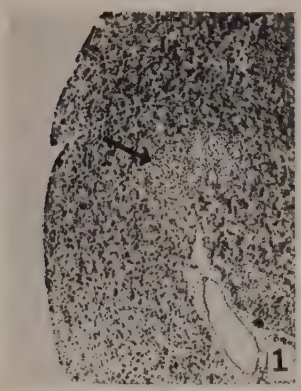
2 Pancreas of hamster killed under the x-ray beam showing islet still clearly demarked.

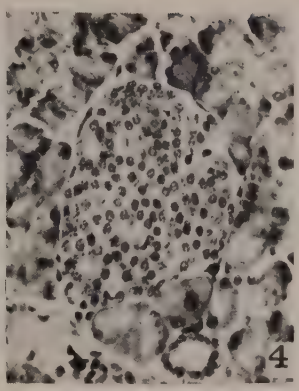
3 Pancreas of hamster given an LD/100 and killed in one week, showing dispersion of islet cells so that it is no longer clearly demarked from the surrounding acinar cells.

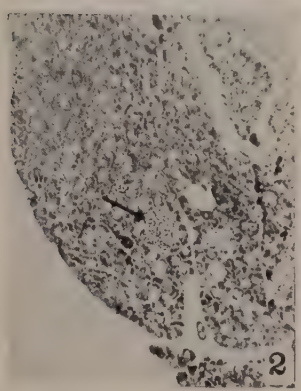
4 From figure 1 enlarged to show uniform islet cells, the surrounding thin connective tissue membrane, and normal acini.

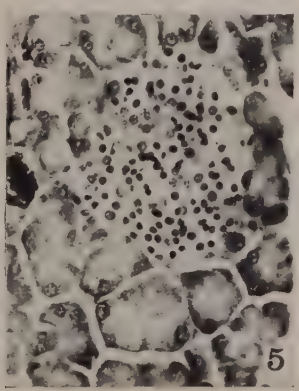
5 From figure 2 enlarged to show more than 50% of the islet cells definitely pyknotic, with some partially pyknotic and still others apparently quite normal. Note breakdown of surrounding connective tissue membrane and essentially normal acinar cells.

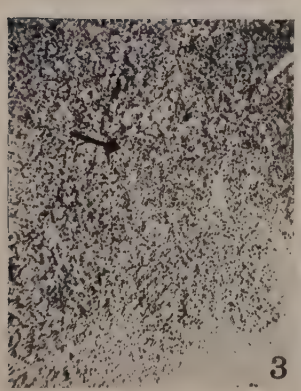
6 From figure 3 enlarged to show low incidence of pyknosis and granular cytoplasm of the islet cells. Note complete loss of surounding connective tissue membrane, and outward dispersion of islet cells.


1


4


2


5


3

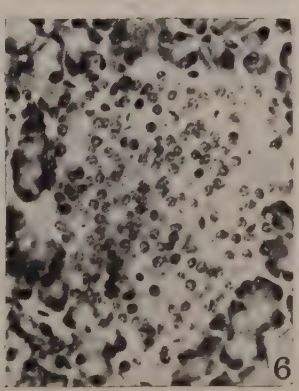

6

PLATE 4

EXPLANATION OF FIGURES

The pancreas of hamsters (2) [2]

7 Hyperemia or dilatation of capillaries leading into an islet of Langerhans.

8, 9 Cytoplasmic vacuoles in acinar cells, and karyorrhexis.

10 Enlarged acinar nucleus, shown near nuclei of normal size.

11–13 Evidences of karyorrhexis in acinar cells.

14, 15 Clumping of chromatin in acinar nuclei of hamsters killed one week after exposure to LD/100 of 1500 r.

16 Isolated nuclei of three sizes, giving evidence of swelling, from same animal as figure 14.

---

[2] Figures 7–13 inclusive are all from hamsters killed under the x-ray beam. Figures 14–16 are from hamsters killed one week after exposure to an LD/100 dose of 1500 r.

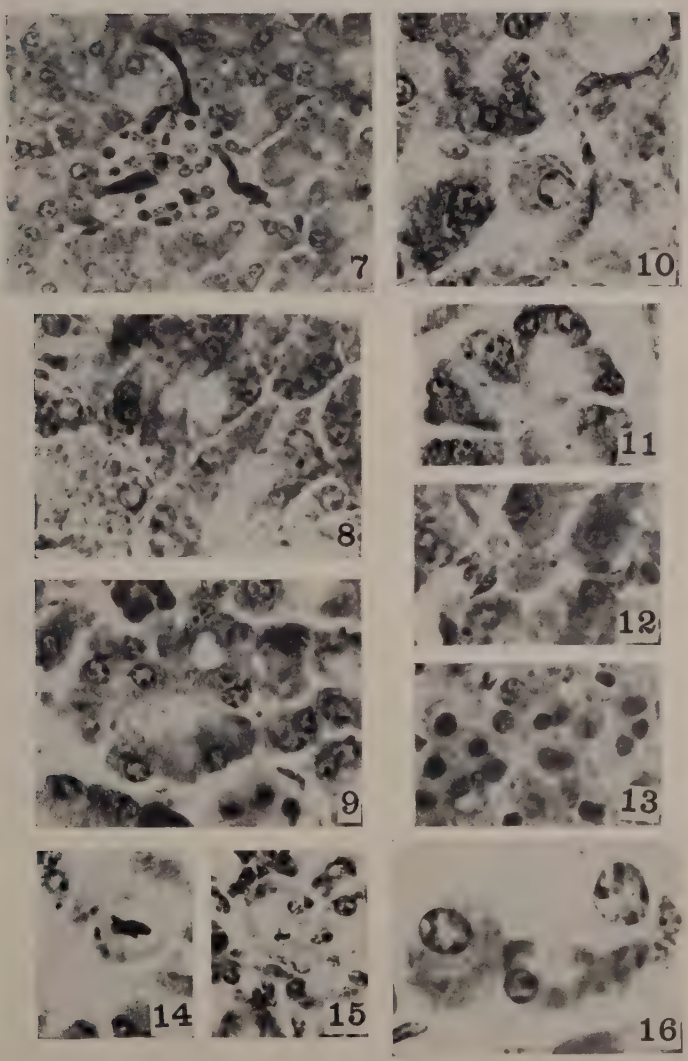

7
10
8
11
12
9
13
14
15
16

PLATE 5

EXPLANATION OF FIGURES

The adrenal gland of hamsters

1 Low power view of control adrenal showing compact medullary cords.

2 Adrenal of hamster killed under the x-ray beam showing dilatation of medullary capillaries and possible swelling of entire medullary region.

3 Adrenal of hamster killed one week following an LD/100 dose of 1500 r showing edemic swelling of medulla and loss of clear cut demarcation between medulla and cortex. (Figs. 1, 2, 3 at same magnification.)

4 Zona fasciculata (from hamster of fig. 2) showing an enlarged nucleus in field of other nuclei of normal size.

5 Medullary region (from hamster of fig. 2) showing enlarged medullary cell nucleus in field of zona reticularis nuclei (to left) and normal sized medullary cell nuclei (to right).

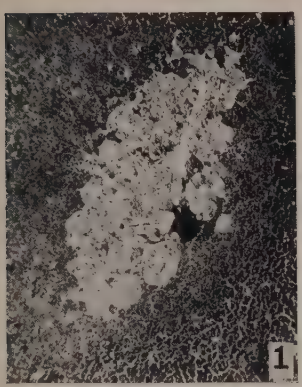
6 Cytoplasmic vacuole in cell of zona fasciculata from hamster of figure 3.

7 Hamster from figure 3 showing region of both zona reticularis (above) and medullary region (below). Note some tendency to pyknosis of reticularis nuclei in contrast with quite normal medullary cell nuclei.

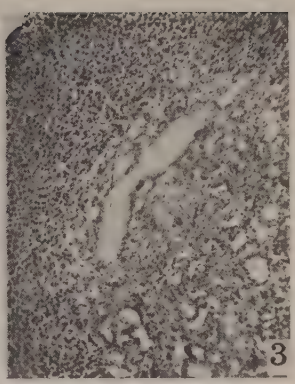
8, 9 Karyorrhexis in medullary cells of hamster dying under the x-ray beam (as figs. 2, 4, 5). Note also breakdown of cytoplasm in figure 8.

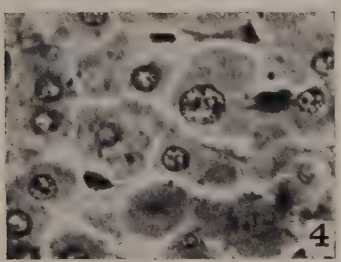
10 Medullary region of hamsters killed one week after exposure to an LD/100 dose of 1500 r (same as figs. 3, 6, 7) showing enlarged nucleus in disintegrating cell, surounded by rather normal medullary cells.

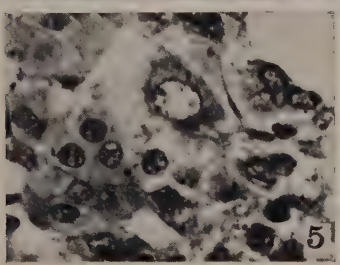
11 Dilated capillary from same hamster as figure 10 showing absence of red blood corpuscles. Surrounding medullary cells appear rather normal.

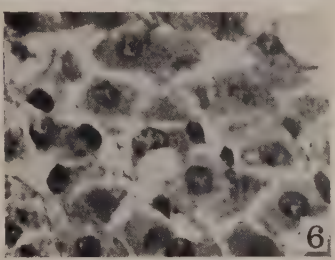

1

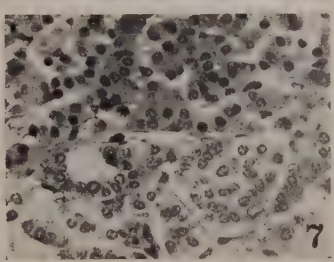

2

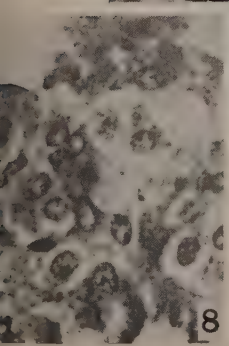

3


4

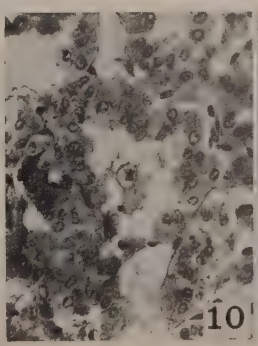

5

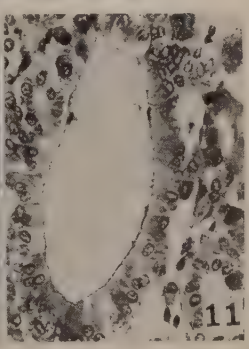

6


7


8


9


10


11

AN ANALYSIS OF THE PHOTOELECTRIC METHOD FOR STUDYING OSMOTIC CHANGES IN CHICKEN ERYTHROCYTES [1, 2]

F. R. HUNTER

Department of Physiology, Florida State University, Tallahassee

FIVE FIGURES

I

Erythrocytes have long been used for permeability studies but in spite of their convenience, considerable care must be exercised in interpreting observations because of the sensitivity of these cells to environmental changes. In most of his writings, Jacobs has emphasized this point. During the past few years the author has been interested in trying to find a method for determining changes in the permeability to nonelectrolytes of erythrocytes which have been subjected to rather marked environmental changes, without too much danger of misinterpretation resulting from other alterations in the cells. Since hemolysis times may be influenced by a variety of factors (see, for example, Jacobs and Parpart, '31), a more sensitive apparatus was constructed (Hunter, '49a) in order to use rates of swelling as a measure of permeability. But it was soon found (Hunter, '49b) that this technique was not too satisfactory, since slight hemolysis occurs under a variety of circumstances which makes it difficult to interpret the results obtained.

[1] This work was supported in part by grants from the Division of Grants and Research, U. S. Public Health Service and the Faculty Research Fund, The University of Oklahoma.

[2] The experimental work was performed in the Department of Zoological Sciences, The University of Oklahoma, Norman, Okla.

Wilbrandt ('41) suggested a variation of this general technique to measure the permeability to non-electrolytes which consisted of equilibrating erythrocytes in a salt solution plus the non-electrolyte. Measurements were then made of the shrinking of the cells as the non-electrolyte leaves the cells into a surrounding salt solution. (Whether or not the rate of entrance and exit of substances into and out of these cells is the same remains to be demonstrated.) The present work represents an attempt to evaluate the records obtained using this technique and to describe the general procedure now being followed in this laboratory to study erythrocytes. Much of the work to be reported does not represent anything which is fundamentally new. However, the characteristics of this particular apparatus using chicken erythrocytes must be established. Furthermore, a fairly complete analysis of the procedures which are followed in this laboratory will simplify the recording of future observations.

II

In the present experiments blood was obtained from a chicken by cardiac puncture under bacteriologically sterile conditions. Heparin was used as an anticoagulant. The blood was centrifuged at 2000–3000 $\times$ g and the plasma and buffy layer were removed. Stock suspensions were made up by mixing equal volumes of erythrocytes and 0.3 M non-electrolyte in Ringer Locke or equal volumes of cells and 0.6 M non-electrolyte in Ringer Locke. An aliquot of one of these stock solutions was added to a salt solution and the rate of shrinking was measured. Salt solutions varying in tonicity from 2 $\times$ Ringer Locke to 0.5 $\times$ Ringer Locke were used.

In each instance 10 cm^3 of the salt solution were placed in the chamber of the photoelectric apparatus previously described, and from 0.06–0.3 cm^3 of one of the stock suspensions was added. It was found that more reproducible readings could be obtained if the cells were added from a specially made pipette which would fill by capillarity to a constant vol-

ume. The temperature was maintained at 37° ± 0.1°C. and bacteriologically sterile techniques were observed except during the 5–10 minutes that the actual readings were being made. The records of the deflection of a Kipp and Zonen torsion string galvanometer were obtained on bromide paper.

A portion of a typical series of records which can be obtained using this shrinking technique is shown in figure 1. A continuous record was obtained during the first 15 seconds and this was followed by an exposure every 15 seconds. To obtain the upper series of curves, equal volumes of erythrocytes and a solution of 0.3 M glycerol in Ringer Locke were equilibrated for at least 15 minutes at 37°C. An aliquot of this stock suspension was added to 10 cm^3 of 2 × Ringer Locke in the apparatus and a record was obtained. This procedure was repeated with a different salt solution until a series of shrinking curves was recorded. The lower series was obtained in the same way except the cells were first equilibrated in a solution of 0.6 M glycerol in Ringer Locke. These cells which contain non-electrolyte are hypertonic to all of the salt solutions used. Thus, when the cells are placed in the salt solutions, water enters and the cells swell. In the more dilute salt solutions (1 × Ringer Locke, for example) some of the cells swell to their hemolytic volume. The ascending portion of some of the curves in figure 1 results from this swelling of all the cells and hemloysis of some of them. As more and more hypotonic environments are selected more and more of the cells swell initially to their hemolytic volume. Spectrophotometric data confirm this point. In one experiment, for example, cells which had been equilibrated in 0.6 M glycerol in Ringer Locke showed 5% hemolysis when placed in 1.25 × Ringer Locke and only 3% when placed in 2 × Ringer Locke.

In order to be certain that the cells which swelled to less than their hemolytic volume after having been suspended in 0.6 M glycerol in Ringer Locke were normal, the following observations were made. After records were obtained similar to those shown in the bottom half of figure 1, about 4 cm^3

of the stock suspension of cells in 0.6 M glycerol in Ringer Locke were centrifuged at approximately 1500 × g, the supernatant fluid was removed and the cells resuspended in 40 cm³ of 2 × Ringer Locke. After standing 10–15 minutes this was centrifuged, the supernatant removed and the cells resuspended in 40 cm³ of Ringer Locke. After standing another 10–15 minutes this suspension was centrifuged, the supernatant removed and 0.3 cm³ of the packed cells was added

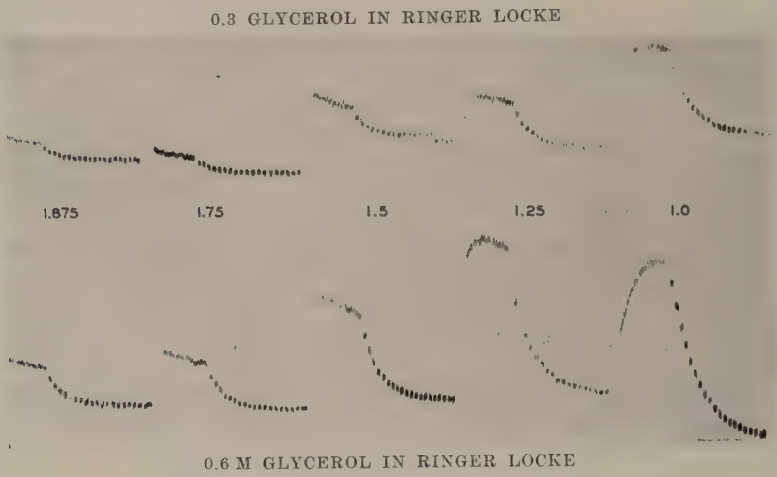


Fig. 1 A series of shrinking curves obtained with chicken erythrocytes at 37°C. Upper series were equilibrated in 0.3 M glycerol in Ringer Locke while the lower series were equilibrated in 0.6 M glycerol in Ringer Locke. The numbers indicate (× Ringer Locke) the concentration of the salt solution in which the shrinking occurred in each case.

to 0.5 cm³ Ringer Locke and another 0.3 cm³ of cells was added to 1 cm³ of 0.3 M glycerol in Ringer Locke. These two cell suspensions then served as new stocks for measuring swelling in 0.3 M glycerol in Ringer Locke and shrinking in 1.5 × Ringer Locke respectively. It was assumed that this washing procedure had removed all of the glycerol from the cells. Figure 2 compares the rates of swelling and shrinking of these washed cells with the original, unwashed stock suspensions. It can be seen that the behavior of these cells is normal. This is not too surprising for, although initially a solution of 0.6 M glycerol in Ringer Locke has an osmotic pres-

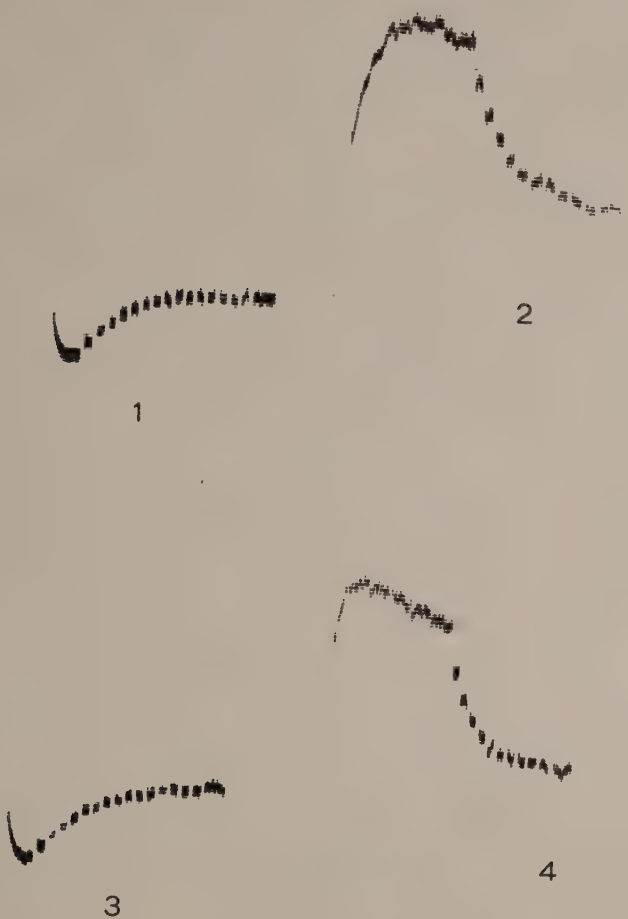


Fig. 2 The effect of previous equilibration in 0.6 M glycerol in Ringer Locke on the osmotic behavior of chicken erythrocytes. 1 — Rate of swelling in 0.3 M glycerol in Ringer Locke of cells incubated in Ringer Locke for 17 hours. 2 — Rate of shrinking in 1.5 × Ringer Locke of cells equilibrated in 0.3 M glycerol in Ringer Locke for 17 hours. 3 — Rate of swelling in 0.3 M glycerol in Ringer Locke of cells incubated in 0.6 M glycerol in Ringer Locke for 17 hours, then washed in 2 × Ringer Locke and resuspended in Ringer Locke. 4 — Rate of shrinking in 1.5 × Ringer Locke of cells incubated in 0.6 M glycerol in Ringer Locke for 17 hours, then washed in 2 × Ringer Locke and resuspended in 0.3 M glycerol in Ringer Locke. Curves 1 and 3 first 5 seconds recorded; curves 2 and 4 first 15 seconds recorded; vertical breaks — 15 second intervals.

sure three times that of the cell, at equilibrium the concentration of glycerol in the cell is only 0.365 M. It might be expected, then, that these cells would not be too different from cells which have swelled in an infinite volume of 0.3 M glycerol in Ringer Locke.

One would predict that as the external medium is made less concentrated, the change in volume required to reach equilibrium would be greater. One would also expect that the more non-electrolyte there is in the cell, the greater would be the volume change. Figure 1 shows that these two predictions are fulfilled. To determine whether or not the observed volume changes agreed with theoretical values based on the van't Hoff-Mariotte law,

$$P_1 (V_1 - b) = P_2 (V_2 - b),$$

the value of b must be determined.

As a first approximation, the dry weight of the cells was obtained in the following way. About 1 cm^3 of cells were centrifuged in an air turbine at 11,000 $\times$ g for 20 minutes. The excess plasma was removed and the centrifuge tubes and cells were weighed. The tubes were then placed in a hot air oven at 110°C. until constant weight was attained. Hematocrit determinations indicated that at least 98% packing was obtained (due to the difference in weights of the two centrifuge heads, the hematocrits were spun at 25,000 $\times$ g). Applying a 2% correction, the amount of water in these cells was 64.5% by weight. This is not very different from the figure obtained by Erickson et al. ('38) of 67%.

To determine whether or not all of the cell water was acting as a solvent, the distribution method employed by other investigators (see, for example, Parpart and Shull, '35) was used. In these experiments a suspension of erythrocytes was added to a solution of 0.3 M ethylene glycol in Ringer Locke and sufficient time was allowed for diffusion equilibrium to be established. The amount of glycol before and after equilibration with the cells was determined using an electrometric technique. Excess dichromate in a strong acid solution reacted with the glycol and the excess was titrated with fer-

rous ion. A correction was applied to the initial glycol solution for oxidizable material in the Ringer Locke and to the equilibrium glycol value for oxidizable material diffusing from the cells. This latter correction was based on a titration of a 4th washing of cells in Ringer Locke prior to equilibration in the glycol solution. The values obtained by this method for the amount of solvent water in the cells varied from 82–93%

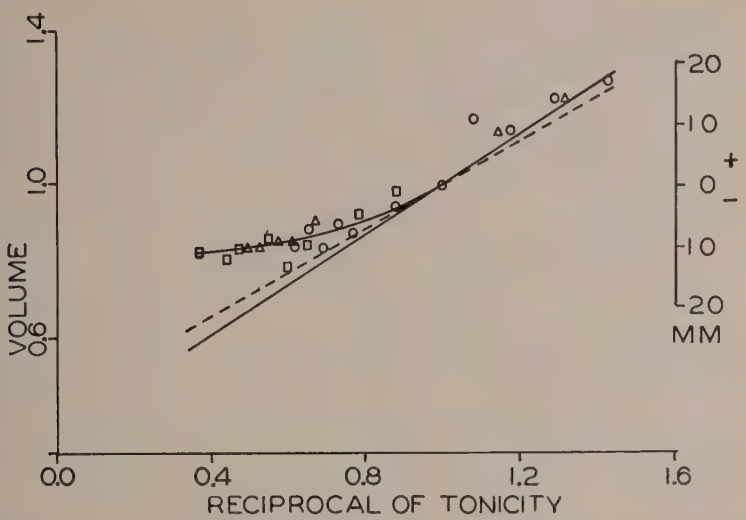


Fig. 3 The relationship between tonicity and volume of chicken erythrocytes as determined by hematocrit measurements (circles and rectangles) and by photoelectric measurements (triangles). Solid straight line is calculated curve using a b value = 0.355; broken line — b = 0.439.

with an average value of 87% of the total cell water as solvent water. This indicates that approximately all of the cell water is acting as a solvent for ethylene glycol which would suggest that the value for b equal to the dry weight might be a reasonable one.

To determine whether or not these cells behaved as perfect osmometers when placed in a series of salt solutions, hematocrit determinations were made. Equal volumes of cells and various dilutions of 10 × Ringer Locke were mixed (at 37°C. in some cases and at room temperature in others without any

gross differences) and hematocrits were run in the air turbine as before. The results are shown in figure 3. The solid straight line was obtained by calculating theoretical volumes as follows: Using 0.355 as the value of b; $P_1 = 1$; and $V_1 = 1$; P_2 was determined by assuming that the total aqueous volume of the system at any time was 1.645 (1 cm³ of salt solution plus 0.645 cm³ of water in 1 cm³ of cells). The total amount of osmotically active material (remembering that the 0.645 cm³ of cell water had 0.645 units of osmotically active material) was 0.645 (contributed by the cell) plus n (where $n =$ the strength of the salt solution in terms of Ringer Locke $= 1$). Thus, when equal volumes of cells and 2 × Ringer Locke were mixed the equation

$$P_1 (V_1 - b) = P_2 (V_2 - b)$$ [3]

$$\text{became } 1(1 - 0.355) = \frac{2.645}{1.645}(V_2 - 0.355)$$

$$V_2 = 0.756.$$

The broken straight line was calculated assuming a b value equal to 0.439 which corrects for 8.4% of the total cell being bound water. The open circles in figure 3 represent the average values of the experimental points. As a further check an additional series of experiments was run under slightly different experimental conditions designed to reduce the experimental error. These results are shown as rectangles in figure 3. Since there is considerable error resulting from the slowness of drainage when packed cells are pipetted, a small amount of plasma was left with the cells after the blood was initially centrifuged. Equal volumes of this cell suspension

[3] Ponder's equation (1) ('50) can be derived from this expression by assuming that $R = 1$ and remembering that: $(V_1 - b) = W$, the water in the cell initially; the ratio of the volume of the surrounding medium to the volume of cell water $= \frac{1}{0.645} = a$; $P_1 = 1$; $V_2 = V$ in Ponder's equation; and Ponder's $T =$ the ratio of the tonicity of the salt solutions used to blood plasma — i.e. for 2 X Ringer Locke $T = 2$ etc. Then $-P_1 (V_1 - b) = P_2 (V_2 - b)$

$$W = \frac{T + 1/a}{1 + 1/a}(V - 1 - W)$$

$$V = W\left(\frac{1}{T + 1/a} - \frac{T}{T + 1/a}\right) + 1$$

and salt solutions were mixed. Hematocrits run on the mixture of cells and Ringer Locke gave a value for the plasma which was left with the cells initially and the calculations were corrected for this.[4] In 4 × Ringer Locke clumps of cells formed which made accurate measurements impossible and in 10 × Ringer Locke all the cells formed a large sticky mass. Forms similar to those observed by Furchgott ('40) were seen in some cases.[5]

These hematocrit data suggest that in the hypotonic range studied and possibly in slightly hypertonic solutions the cells were acting as perfect osmometers. In more hypertonic solutions, however, the volume changes were less than what would be predicted on the basis of the van't Hoff-Mariotte law.

This problem of the behavior of erythrocytes in solutions of various tonicities has been studied extensively. For the most part, mammalian erythrocytes have been used and so the present data are not directly comparable with those of other workers. Two recent papers, however, might be mentioned. Ponder ('50) measured the volumes of human erythrocytes in markedly hypotonic solutions and found good agreement with the van't Hoff-Mariotte law, assuming an R value of 0.9, in solutions which caused little or no hemolysis. In more hypotonic solutions in which hemolysis occurred, there was marked deviation from the law. Ørskov ('46) also using human blood, found good agreement with the van't Hoff-Mariotte law in hypotonic solutions but in hypertonic solutions 15–20% of the cell water acted as if it were "bound." He suggested that perhaps "The salts of the watery phase of the cell exert an augmented osmotic pressure when the hemoglobin concentration is augmented."

[4] In one experiment, for example, the hematocrit of cells in Ringer Locke was 0.280. Thus, 0.280 cm³ of cells plus 0.220 cm³ of Ringer Locke plus 0.5 cm³ of concentrated Ringer Locke solutions were mixed. For 2 × Ringer Locke the calculation becomes:

$$(1)\ (1-0.355) = \frac{(2)\ (0.5) + (1)\ (0.22) + (1)\ (0.28)\ (0.645)}{0.5 + 0.22 + (0.28)\ (0.645)} (V_2 - 0.355)$$

$$V_2 = 0.733.$$

[5] The author is indebted to Mr. Alvin Feldzamen for making this second series of measurements and for making the solvent water determinations.

The present data indicate that chicken erythrocytes, like human erythrocytes, behave as perfect osmometers in slightly hypotonic and slightly hypertonic solutions but in markedly hypertonic solutions they shrink less than would be expected if they were behaving as perfect osmometers. In very hypertonic solutions a marked change in the cells occurs which results in their sticking together.

The next point to be investigated was the relationship between the galvanometer deflections of the apparatus and the volume changes of the cells. Most workers in this field adjust their experimental conditions so that a linear relationship exists between these two variables. To test this point, aliquots of a cell suspension (either cells alone or cells in Ringer Locke) were added to 10 cm^3 of a salt solution in the apparatus and a record taken of the galvanometer deflection. These data are also included in figure 3. The open triangles are average values determined by letting the photograph obtained using Ringer Locke be the origin and measuring on the records obtained with the other salt solutions the distance above or below this point. It can be seen that there is close correlation between volume changes and galvanometer deflections. If the concentration of cells is changed, the amount of light and hence the absolute deflection of the galvanometer will be altered and a different scale will have to be used for the right hand ordinates in the figure. The proportional deflections, however, will be essentially the same over the concentration range used in this laboratory.

Another point to be analyzed concerns the behavior of the cells when a non-electrolyte diffuses from the cells into solutions of different tonicities. With a series of curves such as those shown in figure 1, at least three values could be compared with the theoretical. One might expect that the equilibrium volumes could be plotted against a theoretical curve such as was done in figure 3. It can readily be seen in figure 1, however, that these equilibrium values do not progress in a regular series. Differences in the number of cells in the initial aliquots or slight hemolysis can account for these dis-

crepancies. Another point of comparison might be the volumes immediately after water had first entered the cells as a consequence of the initial osmotic inequality between the cells and their environment. As can be seen from the records, however, this portion of the curve (the first 15 seconds) cannot be measured very accurately. Consequently it was decided

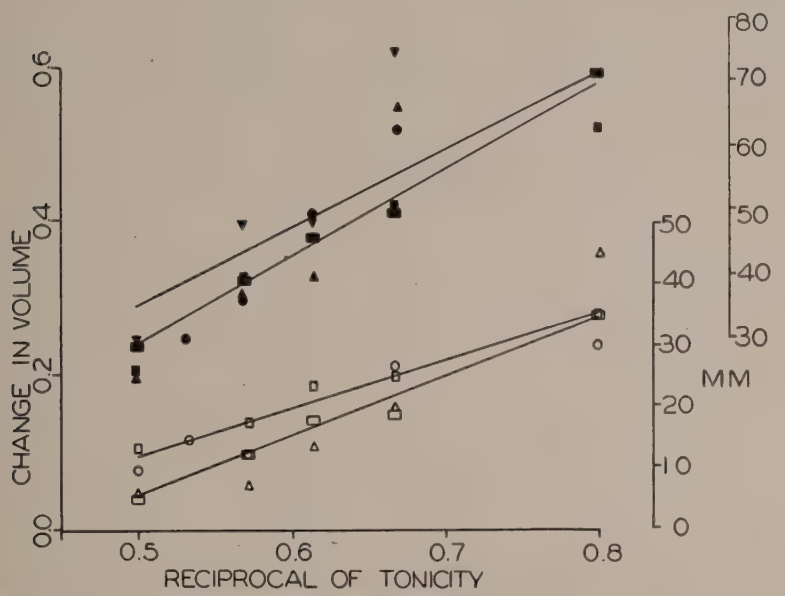


Fig. 4 The relationship between tonicity and volume changes of chicken erythrocytes. For details see text.

to compare the total volume changes in the various salt solutions. Such a comparison is made in figure 4. Once again, the solid lines are calculated. In order to do this it was assumed that the initial entrance of water occurred before any glycerol left the cells. Since glycerol leaves the cells quite slowly in relation to the rate at which water enters, this assumption will introduce only a slight error. In this case P_1 had to be determined for it will be remembered that an equal volume of cells and of 0.3 M (or 0.6 M) glycerol in Ringer Locke had been equilibrated. As was true with the hemato-

crit determinations, the total volume of water in the system is 1.645 cm^3 (1 cm^3 from the solution and 0.645 cm^3 from the cells). At equilibrium in this solution the concentration of glycerol was

$$\frac{0.3}{1.645} = 0.182\ M \text{ (with 0.3 M glycerol) and } 0.365\ M \text{ (with 0.6 M glycerol).}$$

To convert these to osmotically equivalent units (the initial osmotic pressure of the cell $= 1 \sim 0.3$ M glycerol) we divide by 0.3, obtaining 0.61 and 1.22 respectively. For cells which were equilibrated in 0.3 M glycerol in Ringer Locke and were then placed in 2 $\times$ Ringer Locke (considering the volume to be infinite) the calculation becomes:

$$1.61\ (1 - 0.355) = 2\ (V_2 - 0.355)$$
$$V_2 = 0.875.$$

At equilibrium in the salt solution we can use the value previously calculated for figure 3 which was obtained by assuming the external volume was infinite. Under these conditions, all of the glycerol will diffuse from the cells and exert no osmotic effect since it will be infinitely diluted. Thus $\triangle V$ for this case is $0.875 - 0.677 = 0.198$.

It should be remembered, however, that at equilibrium the volumes of the cells in solutions more concentrated than Ringer Locke will be less than 1. This means that in all cases shown in figure 4 the cells are undergoing volume changes in a range where the hematocrit determinations showed that observed volumes were greater than theoretical.

In an attempt to correct for this, two theoretical curves are shown for each glycerol solution (0.6 M above, 0.3 M below). The upper member of each pair of curves was calculated using a value of $b = 0.555$. This value was arbitrarily obtained by drawing a straight line through the experimental points in the slightly hypertonic range in figure 3 and determining the value of b from the intercept. Although Ørskov's suggestion mentioned previously might result in an increasing value of b as the cells were placed in more and more hypertonic solutions, and although the experimental points in figure 3 do seem to depart more and more from the theoretical curve,

an approximation can be made by assuming a constant but larger value of b for tonicities of 2 × to 1.25 × Ringer Locke. The lower member of each pair of curves was plotted by using the volumes indicated by the triangles in figure 3 and correcting the curves as calculated above for the difference between the observed and calculated volumes as shown in figure 3.

With the data plotted in this fashion, it can be concluded that unless hemolysis occurs, there is a linear relationship

TABLE 1

Measurements to indicate the reproducibility of the swelling technique

CELLS SHRUNK (× R. L.)	TIME IN SECONDS TO 50% DEFLECTION						
	Cells equilibrated in 0.3 M glycerol in R. L.			Cells equilibrated in 0.6 M glycerol in R. L.			
2	30	30	45	60	35	35	45
1.875			45			45	
1.75	35	30	30	45	45	45	< 45
1.625	40	45		> 45	45	45	< 45
1.5	> 40	< 45	45	< 60	45	< 60	45
1.25	> 40	45	45	60	< 60	< 75	> 45
1	> 40	45	60	> 75	> 75		> 45
0.875		> 45	60				
0.75	60	> 60	75				
0.625		75					
0.5		> 90					

between galvanometer deflections and volume changes such as those shown in figure 1.

In order to have some basis for comparing shrinking data, measurements were made of several series of curves such as those shown in figure 1 and the times required for one-half the total deflection (cf. Dziemian, '39) are listed in table 1. If one compares the figures in any horizontal column it can be seen that in most instances from day to day using different samples of blood, highly reproducible values can be obtained. (Since measurements were made at 15 second intervals, differences in time less than this must be read from the curves by interpolation.) A comparison of the times in any

vertical column shows in general an increase as the volume changes of the cells increase. A similar comparison can be made between horizontal columns under 0.3 M and 0.6 M glycerol in Ringer Locke.

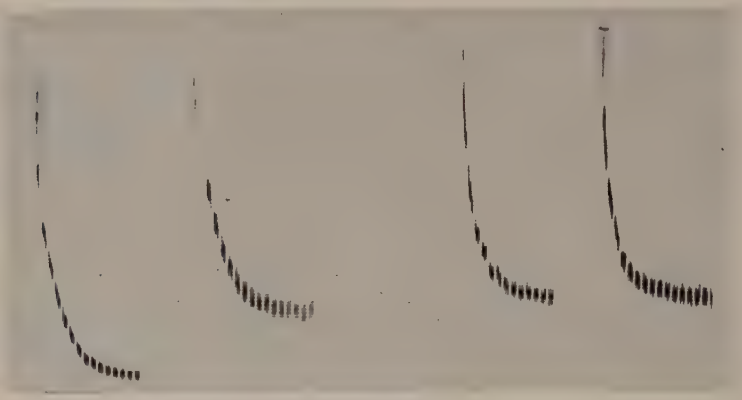


Fig. 5 The rate of shrinking in 1.625 × Ringer Locke of chicken erythrocytes equilibrated in 0.6 M ethylene glycol in Ringer Locke and 0.6 M glycerol in Ringer Locke at 25° and 37°C.

As a final test of the shrinking method, measurements were made at 25°C as well as at 37°C. Lowering the temperature should decrease the rate of penetration (or exit) of molecules, particularly those which do not penetrate too rapidly. It can be seen in figure 5 that the rate of shrinking in 1.625 × Ringer

Locke of cells previously equilibrated in 0.6 M glycerol in Ringer Locke is much slower at 25°C. than at 37°C. The times for one-half the total deflection in two experiments were 40 and < 40 seconds at 37° and 60 and < 75 seconds at 25°C. Even in the case of ethylene glycol which leaves the cells very rapidly there is a decrease in rate at the lower temperature. This can most clearly be seen by comparing the amount the galvanometer moves during each second in the two cases.

III

In light of the preceding analysis and in view of the recent experience in this laboratory, most of which is unpublished, the following methods of analyzing for changes in erythrocytes have become accepted. The rate of hemolysis in an isosmotic solution of a penetrating non-electrolyte is not always a reliable indication of a change in permeability as has been pointed out by other investigators. Exposure to various bacterial toxins, for example, may decrease the time for hemolysis in glycerol very noticeably without altering the rate of swelling or shrinking. Such a change can frequently be correlated with an increase in fragility rather than an increase in permeability. Fragility measurements are made by placing aliquots of the erythrocyte suspensions in a series of tubes containing a decreasing amount of NaCl (1% to 0% in steps of 0.1% usually) or by using the method described by Parpart et al. ('47). The contents of these tubes can then be placed one by one in the permeability apparatus and a record of the galvanometer deflection made, or the tubes can be centrifuged and the amount of hemoglobin in the supernatant fluid measured spectrophotometrically. In most cases the latter technique is preferable for measuring per cents of hemolysis less than 50%.

Swelling measurements obtained by placing cells in 0.3 M non-electrolyte in Ringer Locke are also sometimes difficult to interpret. In the experiments which have been carried out during the past few years in this laboratory the most noticeable change in swelling curves has been an increase in the de-

flection of the galvanometer. As has previously been reported (Hunter, '49b) this increase in deflection may be many fold or it may be slight. In general, the greater the deflection, the more rapid is the deflection. Since some hemolysis is involved in these changes, such measurements give no clear cut indication of a permeability change. Such an increase in amount of galvanometer deflection has been referred to in the past as a "fragility" change. The quotation marks have been used to indicate that a series of measurements other than those involving the usual series of salt solutions has indicated the change. In many experiments an increase in fragility as measured in the usual manner is paralleled by an increase in "fragility" as indicated by swelling measurements. However, in some cases a marked increase in fragility has been observed with little or no change in "fragility." This suggests the possibility that these two changes may not be the same — quantitatively if not qualitatively. This being the case, in the future the term frailty will be used as a measure of the tendency of the cells to hemolyze following volume changes in a solution which at equilibrium would be expected to be isosmotic. Although it is undesirable to add new terms to an already overburdened literature, this term, frailty, will be very useful since it will be associated with a particular kind of measurement that is quite different from fragility measurements. The work of various investigators suggests that changes in fragility and possibly in frailty are assocated with shifts in the amount of cations within the cells.

In previous publications (Hunter, '49b, for example) the values of per cent hemolysis which were given as an indication of frailty changes were too high because they were uncorrected for hemolysis which had occurred in the stock suspension prior to making the frailty measurements. Although it would seem to be a simple matter to determine this hemolysis in the stock suspension, several difficulties exist. The most direct approach would be to centrifuge the stock suspensions and determine the amount of hemoglobin in the supernatant fluid. But when there was any appreciable hemoly-

sis this method always gave low values for the obvious reason that the volume of fluid outside the cells was small and consequently after the hemoglobin had diffused to equilibrium there was still considerable hemoglobin left in the hemolyzed cells. When a small aliquot of these stock cells were placed in a relatively large volume (10 cm^3, for example) the rest of the hemoglobin diffused out of the hemolyzed cells immediately.

Another method would be to transfer an aliquot of the stock to 10 cm^3 of some solution and determine the amount of hemolysis but the choice of the suspending solution is important. In some instances the cells were sufficiently fragile so that when they were placed in Ringer Locke not only did the hemoglobin diffuse out of the cells which had hemolyzed in the stock but apparently additional cells hemolyzed. As might have been expected on the basis of an increase in frailty, some additional cells hemolyzed when placed in 0.3 M gycerol in Ringer Locke. The least hemolysis in many cases was observed when the cells were placed in 2 $\times$ Ringer Locke. Consequently, this solution will be used in the future to correct the frailty readings.

Shrinking measurements should indicate whether or not the permeability of the cells has changed. In general, equilibrating cells in 0.6 M glycerol in Ringer Locke and measuring the shrinking in 1.5 or 1.75 $\times$ Ringer Locke should give the most satsfactory results. An indication of a fragility change can be gotten by determining the amount of hemolysis which occurs when the cells shrink in less hypertonic solutions. Presumably the hemolysis would occur when the cells swell initially prior to their shrinking. An increase in hemolysis under these circumstances should parallel an increase in hemolysis in ordinary fragility measurements.

SUMMARY

1. The dry weight of chicken erythrocytes is 35.5% of the total weight of the cells.

2. Measurements of solvent water in these cells indicate that 82 to 93% of the cell water acts as a solvent for ethylene glycol.

3. In slightly hypotonic and in slightly hypertonic salt solutions, chicken erythrocytes behave as perfect osmometers. In markedly hypertonic solutions these cells shrink less than would be predicted from van't Hoff-Mariotte's law.

4. Using a photoelectric apparatus, there is an approximately linear agreement between galvanometer deflection and volume change.

5. The term frailty is introduced to designate the tendency of erythrocytes to hemolyze following volume changes in a solution which at equilibrium should be isosmotic.

6. An analysis was made of various methods used to study the osmotic behavior of chicken erythrocytes. In general:

(a) A decrease in hemolysis times frequently is associated with an increase in fragility.

(b) Swelling measurements indicate an increase in frailty when the galvanometer deflection increases.

(c) Shrinking measurements appear to give a method for measuring changes in permeability. It is recommended that cells be equilibrated in 0.6 M non-electrolyte in Ringer Locke and then the shrinking be measured in 1.5–1.75 × Ringer Locke.

LITERATURE CITED

DZIEMIAN, ARTHUR J. 1939 The lipid content and the permeability of the erythrocyte. J. Cell. and Comp. Physiol., *14:* 103–116.

ERICKSON, BETTY NIMS, HAROLD H. WILLIAMS, SAMUEL S. BERNSTEIN, IRA AVRIN, ROBERT L. JONES AND ICIE G. MACY 1938 The lipid distribution of posthemolytic residue or stroma of erythrocytes. J. Biol. Chem., *122:* 515–528.

FURCHGOTT, ROBERT F. 1940 Observations on the structure of red cell ghosts. Cold Spring Harbor Symp. Quant. Biol., *8:* 224–232.

HUNTER, F. R. 1949a A modified permeability apparatus for permeability studies. Science, *109:* 119–121.

———— 1949b "Control" erythrocytes II. J. Cell. and Comp. Physiol., *33:* 199–214.

JACOBS, M. H., AND ARTHUR K. PARPART 1931 Osmotic properties of the erythrocyte. II. The influence of pH, temperature, and oxygen tension on hemolysis by hypotonic solution. Biol. Bull., *60:* 95–119.

ØRSKOV, S. L. 1946 The volume of the erythrocytes at different osmotic pressures, further experiments on the influence of lead on the permeability of cations. Acta. Physiol. Scand., *12:* 202–212.

PARPART, ARTHUR K., JOHN R. GREGG, PHILIP B. LORENZ, ETHYL R. PARPART AND AURIN M. CHASE 1947 Whole blood preservation; a probem in general physiology. An *in vitro* analysis of the problem of blood storage. J. Clin. Invest., *26:* 641–654.

PARPART, ARTHUR K., AND JOHN C. SHULL 1935 Solvent water in the normal mammalian erythrocyte. J. Cell. and Comp. Physiol., *6:* 137–150.

PONDER, ERIC 1950 Tonicity-volume relations in partially hemolyzed hypotonic systems. J. Gen. Physiol., *33:* 177–193.

WILBRANDT, W. 1941 Zur Permeationskinetik rasch eindringen der Substanzen an Erythrocyten. Pfluger's Arch., *245:* 1–21.

THE ADSORPTION OF OXYGEN FROM BUFFER SOLUTIONS BY CELLS OF ESCHERICHIA COLI B/r [1]

M. LAURANCE MORSE,[2] ARTHUR W. BURKE, JR.,[3] AND WILLIAM T. BURNETT, JR.

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

TWO FIGURES

Hollaender, Stapleton and Martin ('51) have demonstrated that a reduction in the oxygen tension before x irradiation of suspensions of *Escherichia coli* B/r will increase the cell survival. Recently we have been examining the relationship of the cell survival of x-irradiated suspensions of *E. coli* to oxygen concentration (Burnett et al., '51, '52). Oxygen has been shown to affect the production of radiochemical products in aqueous systems (see e.g., Gray, '51) and since the radicals which are formed in these reactions are probably toxic, the effect of oxygen on the cell survival of irradiated cell suspensions may depend upon localization of oxygen within or upon the bacterial cells. The possibility of a localization of oxygen affecting irradiated cell survival has led to a study of the ability of *E. coli* to adsorb oxygen from phosphate buffer suspensions.

EXPERIMENTAL METHODS AND RESULTS

Cells of *E. coli* B/r were cultured for 20–24 hours under aeration at 37°C. in 30–50 ml volumes of Difco nutrient broth (0.8%) containing NaCl (0.5%), harvested by centrifugation,

[1] Work performed under Contract No. W-7405-Eng-26 for the Atomic Energy Commission.

[2] Present address: Department of Genetics, University of Wisconsin, Madison 6, Wisconsin.

[3] Present address: St. Louis University, Institute of Biophysics, St. Louis, Mo.

washed once in M/15 phosphate buffer (pH 6.8), and resuspended in 1/20 to 1/30 original culture volume in M/15 phosphate buffer, which effected a 500-fold dilution in broth concentration.

TABLE 1

The association of oxygen with cells of E. coli B/r at various oxygen concentrations of the suspending medium (2°C.)

EXPERIMENT	OXYGEN CONCENTRATION OF BUFFER		CELL TITER	OXYGEN ASSOCIATED WITH CELLS X/N
	Initial [1]	Equilibrium [2] C		
	mg/l	*mg/l*	$\times 10^{9}$ *cells/ml*	$\times 10^{-13}$ *mg/cell*
1	2.1	1.3	6.7	1.2
3	2.3	1.5	5.7	1.4
3	3.2	1.1	5.7	0.4
2	4.6	3.2	6.4	2.2
3	5.3	3.2	5.7	3.7
3	7.0	4.9	5.7	3.7
2	7.6	5.8	6.4	2.8
2	10.0	8.0	6.4	3.1
4	12.0	10.5	4.8	3.1
3	12.3	10.0	5.7	4.0
1	12.4	9.3	6.7	4.6
2	12.4	9.8	6.4	4.1
3	23.8	20.0	5.7	6.7 [3]
1	23.9	20.9	6.7	4.5
2	24.6	22.0	6.4	4.1
4	24.1	22.3	4.8	3.7
2	32.0	29.4	6.4	4.1
1	50.0	46.2	6.7	5.7 [3]

[1] Before addition of cells.

[2] Immediately after sedimentation of cells (volume corrected for loss due to cell volume).

[3] Not included in calculations in table 2.

The concentrated cell suspensions were then deoxygenated by passing dry nitrogen (99.996%, Linde) through them for 30-60 minutes at ice-bath temperature (2°C.). Solutions of different oxygen concentrations were prepared by passing oxygen-nitrogen mixtures (from 220-cu. ft. gas cylinders) through 250-ml volumes of M/15 phosphate buffer at 2°C. The aerations were made in 500-ml gas washing bottles pro-

vided with a ground glass stoppered side arm through which samples could be easily removed with a pipette.

Equal volumes of a buffer containing dissolved oxygen were added to each of two centrifuge tubes in an ice bath. A measured quantity of a deoxygenated cell suspension was then added to one of the tubes and the contents of both tubes layered with mineral oil and held in an ice bath (2°C.) for a given period of time. The number of cells added to the centrifuge tube was determined from platings (on nutrient agar) of a countable dilution of the nitrogen aerated cell suspension. After the holding period (20–30 minutes), the tubes were spun in a refrigerated centrifuge (1°–3°C.) for 20–30 minutes. The clear, cell-free supernatant ($< 1\%$ original cell titer) from the tube of sedimented cells and the buffer from the control tube were transferred under oil to beakers, and the oxygen content of each determined by the Schutzenberger method (Scott, '39).

The oxygen concentration of the buffers (2–50 mg/liter) was lowered in every case by the addition of the bacterial cells (table 1). The difference between the oxygen concentrations of the buffer which had contained suspended cells and the buffer control, without cells, is attributed to oxygen which had become associated with the cells.

In occasional tests the sedimented cells were resuspended in oxygen-free buffer under oil and the suspension titrated for oxygen. The value of the oxygen concentration was the same as the difference in the oxygen concentration between the buffer from which the cells had been sedimented and the control buffer, within the limits of the titration method. In experiment 5 the cells were resuspended in their original buffer following titration of the buffer with sodium hydrosulfite and sufficient hydrosulfite was added to complete the titration for oxygen. Table 2 shows the results of the experiment. Since the precision of methods under our experimental conditions limits the reliability of the values of cell-associated oxygen for low cell titers, even at a buffer oxygen concentration of 9 mg/l, we did not investigate fur-

ther the variation of cell-associated oxygen concentration with respect to various cell titers.

The concentration of oxygen associated with the cells is a function of the equilibrium concentration of the buffer oxygen

TABLE 2

The association of oxygen with cells of E. coli, B/r at various cell titers (2°C.)

CELL TITER (cells/ml)	6.4×10^9	1.6×10^9	0.8×10^9
Oxygen concentration (mg/l)			
A. Buffer control	8.9	9.3	9.3
B. Supernatant and cells resuspended in supernatant	8.9	9.9	9.3
C. Supernatant	7.9	9.3	8.9
Oxygen associated with cells (mg/cell)			
Procedure 1: A-C	1.0	0.0	0.4 [1]
Procedure 2: B-C	1.0	0.6	0.4
Oxygen associated with cells (mg/cell)	1.6×10^{-13}	3.8×10^{-13}	5.0×10^{-13}

[1] The absolute number of cells used was 6×10^{10}. The oxygen associated with these cells required only a net volume of 0.1 ml of our standard sodium hydrosulfite solution. Thus the precision in this case was low due to errors in the burrette readings.

(fig. 1). Langmuir ('16, '17, '18) has characterized the adsorption of gases and liquids on the surfaces of liquids and solids by a similar curve. Plots (fig. 2), according to the Langmuir adsorption Isotherm,[4]

$$\frac{p}{q} = \frac{1}{k_1 k_2} + \frac{p}{k_2}$$

where

p = oxygen pressure
q = amount of oxygen adsorbed per unit of surface
k_1 and k_2 are constants of the system (Langmuir, '18, p. 1384; Glasstone, '46, p. 1199),

indicate that this function is applicable to our data. Table 3 gives the constants, k_1 and k_2, for 4 experiments, calculated from the data within the range of oxygen concentration of 1 and 30 mg/liter.

[4] Here we take the simpler form of the adsorption isotherm which applies only to adsorption of monomolecular layers, rather than the more elaborate general equations developed by Langmuir ('32).

The adsorption of oxygen by cells of *E. coli* B/r, at 2°C., is approximated from the weighted averages of the values in table 3 by

$$\frac{X}{N} = \frac{1.22 \times 10^{-13}\ C}{1.00 + 0.278\ C}$$

where

X = quantity of oxygen adsorbed in mg.
N = number of cells
C = equilibrium concentration of buffer oxygen in mg/liter.

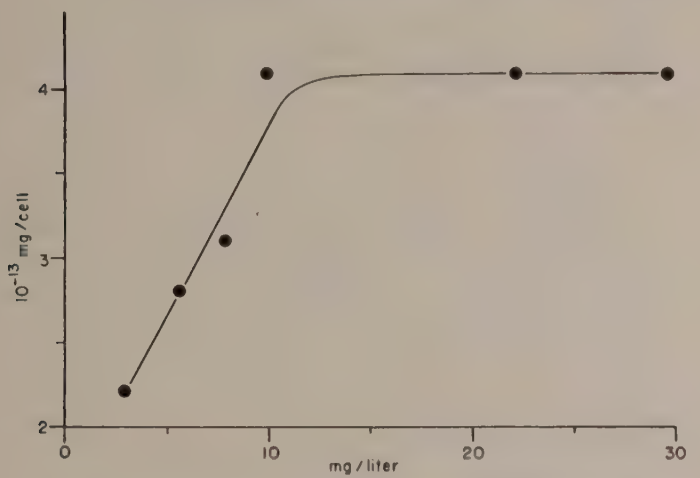


Fig. 1 Oxygen associated with cells as a function of equilibrium oxygen concentration of buffer.
Data of experiment 2: cell titer of experimental tubes = 6.4×10^9 cells/ml, each tube containing 75 ml of buffer; tubes held in ice bath for 30 minutes, centrifuged (4000 r.p.m.) at 2°C. for 20 minutes.

The cells which were noncolony forming, but which probably adsorbed oxygen, are not taken into consideration in this approximation.[5]

The adsorption of oxygen by *E. coli*, at 2°C. for values of $C < 1$ mg/liter or > 30 mg/liter does not conform to the Langmuir Adsorption Isotherm under our experimental con-

[5] Visible colonies were formed by 74–94% of the cells. A numerical average of 24 determinations (total count made with Petroff-Hauser bacteria counting cell) for 7 cultures was 84%. Less than 1% of the cells were clumped. Hegarty and Weeks ('40) have shown that exposure of *E. coli* (from stationary phase of growth) to 2°C. may kill 10–15% of the cells.

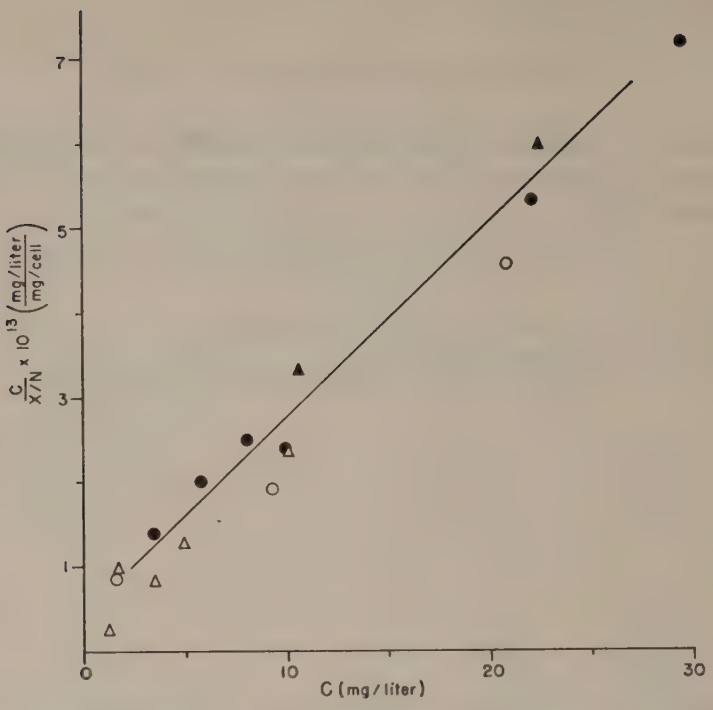


Fig. 2 Plot of data from 4 experiments, according to Langmuir Adsorption Isotherm of the form $\frac{C}{X/N} = \frac{1}{k_1 k_2} + \frac{C}{k_2}$

where

C is the equilibrium concentration of oxygen (buffer);
X/N, adsorbed oxygen/cell;
k_1, k_2, are constants, $\left[\left(\frac{1}{k_1 k_2}\right)\right.$ is the intercept, $\left.\left(\frac{1}{k_2}\right)\right]$ is the slope of the line.

Symbols: ○ = Experiment 1 ● = Experiment 2
△ = Experiment 3 ▲ = Experiment 4

TABLE 3

The adsorption of oxygen by E. coli B/r at 2°C. Constants according to Langmuir Adsorption Isotherm

EXPERIMENT	NO. OF POINTS	k_1 [1]	k_2 [1]
1	3	0.359	5.26×10^{-13}
2	6	0.277	4.76×10^{-13}
3	5	0.255	3.57×10^{-13}
4	2	0.230	4.35×10^{-13}
	Weighted average [2]	$k_1 = 0.278$;	$k_2 = 4.38 \times 10^{-13}$
			$k_1 k_2 = 1.22 \times 10^{-13}$

[1] Slope $\left(\frac{1}{k_2}\right)$ and intercept $\left(\frac{1}{k_1 k_2}\right)$ calculated by the method of least squares in experiments 1, 2, 3; by analytical formulas in experiment 4.

[2] Numerical average of constants, weighted by the number of points in each experiment.

ditions. Although the system is not a "closed" one, the slow rate of oxygen diffusion through the layers of mineral oil does not appreciably affect the results in the intermediate range of oxygen concentration. However, diffusion of oxygen in mineral oil introduces an error at very low concentrations of oxygen. Further, the cell suspensions were deoxygenated by bubbling with nitrogen. Nitrogen aeration is less efficient than removal of oxygen from buffer solutions by certain chemicals (Burnett et al., '52). A residual adsorption of oxygen would skew the adsorption isotherm at low values of C. Also, the error in titration of oxygen by the Schutzenberger method is a limiting factor at values of C less than 1 mg/liter.

At $C > 13$ mg/liter (equilibrium concentration of oxygen in phosphate buffer with air at 2°C.), diffusion of oxygen through the mineral oil layer is out of, rather than into, the system. Also, it may be possible that oxygen is adsorbed by *E. coli* B/r in multimolecular layers at large values of C. The adsorption isotherm of the form used here would not apply to a multimolecular adsorption (Brunauer, '43; Glasstone, '46).

DISCUSSION

A simple calculation shows that an *E. coli* B/r cell adsorbs 7.8×10^6 molecules of oxygen.[6] Therefore, the number of

[6] Microscopic observations indicated that the dimensions of the average cell are $2.5 \times 0.5 \times 0.5\ \mu$, and since the cell may be considered as a right circular cylinder capped by two hemispheres, the surface area would be

total area of cell = area of right circular cylinder + area of sphere

$$A = \pi Dh + \pi D^2$$
$$= 3.92 \times 10^{-8} \text{ sq. cm.}$$

It has been calculated (Langmuir, '18, p. 1391) that the number of molecules of oxygen which would be adsorbed onto a plane surface (solid) as a monomolecular layer is 0.77×10^{-15} molecules/sq. cm of surface. Therefore, if we assume the bacterial surface is "smooth," there would be

$$(3.92 \times 10^{-8} \text{ sq. cm/cell}) \times (0.77 \times 10^{-15} \text{ molecules/sq. cm})$$
$$= 3.02 \times 10^{7} \text{ molecules of oxygen/cell.}$$

The actual number of oxygen molecules adsorbed at saturation by a cell (experiment 2 taken as typical, fig. 1) is

$$(4.1 \times 10^{-13} \text{ mg/cell}) \times \frac{(6.06 \times 10^{23} \text{ molecules})}{32 \text{ g}} \times (10^{-3} \text{ g/mg})$$
$$= 7.76 \times 10^{6} \text{ molecules of oxygen/cell.}$$

molecules of oxygen adsorbed per cell, as estimated, represents 26% of the maximum number of molecules calculated to cover the cell wall as a monomolecular liquid layer (3.0×10^7 molecules/cell). Langmuir ('18, p. 1391) found that the number of adsorbed molecules of carbon monoxide, argon, oxygen, methane, and nitrogen on glass or mica is many times less than the number of molecules calculated to cover completely the adsorbing surface. Oxygen was the least adsorbed of these gases, and the number of oxygen molecules actually adsorbed (at 90°K.) on glass and mica was 14 and 11% of the maximum number of molecules calculated, respectively, to cover the measured surfaces as monomolecular liquid layers.

It is not unlikely that some oxygen diffuses into the bacterial cell and is adsorbed on surfaces inside the cell. Then, there would be: oxygen dissolved in the buffer-cell environs, oxygen adsorbed on the cell walls, oxygen dissolved inside the cells, and oxygen adsorbed on surfaces inside the cells. The oxygen adsorbed inside the cell would probably be distributed throughout the cell, depending upon the availability of adsorbing surfaces. To compare the amounts of dissolved and adsorbed oxygen per cell, we may assume that oxygen is dissolved throughout the entire volume of the cell and in equilibrium with the buffer oxygen, and that the solubility of oxygen is the same in buffer and cell. There would then be, at saturation of adsorption, 1.1×10^5 molecules of dissolved oxygen/cell,[7] as compared with 7.8×10^6 molecules of ad-

[7] Volume of the cell = volume of a right circular cylinder + volume of the two hemispheres

$$V = \pi r^2 h + 4/3\pi r^3$$
$$= 0.458\ \mu^3.$$

At saturation of adsorption the oxygen concentration of the buffer (experiment 2 taken as typical) is

$$(13\text{ mg/liter}) \times \frac{(6.06 \times 10^{23}\text{ molecules})}{32\text{ g}} \times (10^{-12}\text{ ml}/\mu^3) \times (10^{-3}\text{ liter/ml})$$
$$\times (10^{-3}\text{ g/mg}) = 2.46 \times 10^5\text{ molecules of oxygen}/\mu^3$$

Therefore the dissolved oxygen concentration per cell is

$$(2.46 \times 10^5\text{ molecules}/\mu^3) \times (0.458\ \mu^3/\text{cell})$$
$$= 1.13 \times 10^5\text{ molecules/cell.}$$

sorbed oxygen/cell. Hence, there is at least 70 times as much adsorbed as there is dissolved oxygen per cell at saturation of adsorption. Assuming that there is no diffusion of oxygen into the cell, the concentration of adsorbed oxygen on the cell surface is still much higher than the concentration of oxygen dissolved in the buffer environs.

The specific surface of oxygen adsorbent in *E. coli* B/r at 2°C. (calculated at saturation of adsorption of a monomolecular liquid layer) is 1.0×10^{-8} sq. cm/cell, or 2.0 sq. m/g of cells.[8] The specific surface of nitrogen adsorbent for cells of *Azotobacter,* a free living, nitrogen-fixing soil organism, has been calculated to be 3.4 sq. m/g (Brunauer and Emmett, '37; Lineweaver, '38). The specific surface of adsorption of oxygen for cells of *E. coli* B/r is thus the same order of magnitude as the specific surface of nitrogen adsorption for cells of *Azotobacter.*[9]

Oxygen has been shown to affect the production of radiochemical products in aqueous systems (see, e.g., Gray, '51). If the H radical produced by the net ionization of irradiated water, $H_2O \overset{M}{\longrightarrow} H + OH$, combines with molecular oxygen, the highly reactive HO_2 radical is formed: $H + O_2 \rightarrow HO_2$ (Weiss, '44). The localization with respect to "vital targets," of the oxygen which enters into the formation of the HO_2 radical thus appears to be critical. Our calculations indicate

[8] The specific surface area of adsorption is

$$\frac{7.76 \times 10^{6} \text{ molecules of adsorbed oxygen/cell}}{0.77 \times 10^{15} \text{ molecules of oxygen/sq. cm}} \times (10^{-4} \text{ sq. m/sq. cm})$$

$$= 10.0 \times 10^{-13} \text{ sq. m/cell} = 1 \times 10^{-8} \text{ sq. cm/cell.}$$

Volume of cell $= 4.58 \times 10^{-13}$ cc,
specific gravity of cell $= 1.1$ g/cc, and
therefore weight of cells $= 1.1 \text{ g/cc} \times 4.58 \times 10^{-13}$ cc/cell
$= 5.0 \times 10^{-13}$ g/cell.

The specific surface area of oxygen adsorption per gram of cells is

$$\frac{10.0 \times 10^{-13} \text{ sq. m/cell}}{5.0 \times 10^{-13} \text{ g/cell}}$$

$= 2.0$ sq. m/g of cells.

[9] Attention is called here to the experiments of W. Hene (see Brunauer, '43, pp. 11–12) which indicate that oxygen and nitrogen are adsorbed on charcoal at 15°C. in approximately equal volumes (at low pressures, Brunauer, ibid., p. 287). The adsorption potentials of charcoal for oxygen and nitrogen are nearly the same, i.e., 4450 and 4320 cal., respectively (Brunauer, ibid., p. 111).

that, on the basis of relative concentrations alone, adsorbed oxygen is more critical than dissolved oxygen in determining the characteristics of the x-ray inactivation of cells as a function of oxygen tension. A paper is now in preparation on the role of oxygen in the x-ray inactivation of *E. coli* B/r cells in phosphate buffer suspensions.

It is not likely, at 275°K., that the adsorption measured in these experiments is simply of the van der Waals type. The reversibility of the adsorption indicates, however, that oxygen adsorption at 2°C. by *E coli* B/r is not the chemisorption measured in nonliving systems. The nature and location of the oxygen-adsorbing surfaces of the bacterium cannot be deduced from these experiments, but the surface of any membrane, enzyme, or molecule whose primary and secondary valence forces permit the adsorption of oxygen must be considered potential sites of oxygen adsorption.

SUMMARY

Cells of the bacterium, *Escherichia coli* B/r, aerobically cultured and suspended in phosphate buffer at 2°C. adsorb dissolved oxygen from the buffer in agreement with the approximation

$$\text{adsorbed oxygen, mg/cell} = \frac{1.22 \times 10^{-13}\ C}{1.00 + 0.278\ C}$$

where C = the equilibrium concentration of oxygen in the buffer, (mg/liter) within the limits of 1 and 30 mg/liter. The nature of this oxygen adsorption is discussed, and the probable role of oxygen adsorption in the x-ray inactivation of cells is suggested.

ACKNOWLEDGMENT

The authors wish to express their gratitude to members of the Biology Division for their generous help and criticism in the preparation of this manuscript.

LITERATURE CITED

BRUNAUER, STEPHEN 1943 The adsorption of gases and vapors, Vol. I, Physical adsorption. Princeton University Press, Princeton, N. J.

BRUNAUER, STEPHEN, AND P. H. EMMETT 1937 The use of low temperature van der Waals adsorption isotherms in determining the surface areas of various adsorbents. J. Am. Chem. Soc., *59:* 2682–2689.

BURNETT, WILLIAM T., JR., G. E. STAPLETON, M. L. MORSE AND ALEXANDER HOLLAENDER 1951 Reduction of X-ray sensitivity of *Escherichia coli*, B/r by sulfhydryl compounds, alcohols, glycols and sodium hydrosulfite. Proc. Soc. Exp. Biol. Med., *77:* 636–638.

BURNETT, WILLIAM T., JR., M. LAURANCE MORSE, ARTHUR W. BURKE, JR., AND ALEXANDER HOLLAENDER 1952 Reduction of the X-ray sensitivity of *Escherichia coli* by sodium hydrosulfite and certain other inorganic sulfur compounds. J. Bact., *63:* 591–595.

GLASSTONE, SAMUEL 1946 A textbook of physical chemistry, 2nd ed., D. Van Nostrand Co., Inc., New York, N. Y.

GRAY, L. H. 1951 Biological actions of ionizing radiations. Progress in Biophys. and Biophysical Chem. Vol. 2, ed. J. A. V. Butler and J. T. Randall, F. R. S., Pergamon Press, Ltd., London.

HEGARTY, C. P., AND O. B. WEEKS 1940 Sensitivity of *Escherichia coli* to cold shock during the logarithmic growth phase. J. Bact., *39:* 475–484.

HOLLAENDER, ALEXANDER, G. E. STAPLETON AND F. L. MARTIN 1951 X-ray sensitivity of *E. coli* as modified by oxygen tension. Nature, *167:* 103–104.

LANGMUIR, IRVING 1916–1917 The constitution and fundamental properties of solids and liquids, Part I. J. Am. Chem. Soc., *38:* 2221–2295. Part II. J. Am. Chem. Soc., *39:* 1848–1906.

———— 1918 The adsorption of gases on plane surfaces of glass, mica and platinum. J. Am. Chem. Soc., *40:* 1361–1403.

———— 1932 Vapor pressures, evaporation, condensation, and adsorption. J. Am. Chem Soc., *54:* 2798–2832.

LINEWEAVER, HANS 1938 The solubility and chemical and physical adsorption of nitrogen gas in *Azotobacter* cells. J. Biol. Chem., *122:* 549–567.

SCOTT, W. W. 1939 Scott's standard methods of chemical analysis, Vol. I, 5th ed., edited by N. H. Furman. D. Van Nostrand Co., Inc., New York, N. Y.

WEISS, JOSEPH 1944 Radiochemistry of aqueous solutions. Nature, *153:* 748–750.

PENETRATION OF THE INTACT FROG NERVE TRUNK BY POTASSIUM, SODIUM, CHLORIDE AND SUCROSE

ABRAHAM M. SHANES AND MORRIS D. BERMAN

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Federal Security Agency, Bethesda, Maryland

FIVE FIGURES

INTRODUCTION

Bioelectrical changes induced in frog nerve by a large variety of experimental conditions recently have been demonstrated to be closely correlated with alterations in the distribution of sodium and potassium ions (Shanes, '51b, '52). Preliminary analysis of the kinetics of potassium movement, based on the epineurium (sheath) as the major diffusion barrier and on available data on sodium and chloride diffusibility (see also Fenn et al., '34), while giving a satisfactory description of the temporal characteristics of the electrical changes, predicts magnitudes which are too great (Shanes, '52).

The present study was undertaken to determine the extent to which previous assumptions regarding the diffusion characteristics of the system are valid. The current controversy regarding this problem rests almost exclusively on indirect evidence (see Lorente de Nó, '52; and Crescitelli, '51, for earlier references and discussion). The direct data to be presented indicate, in the light of more recent results to be presented in detail in a later communication, that the sheath controls the diffusion process in frog nerve *in vitro*.

The results to be discussed suggest at least two extracellular phases contributing to the uptake or release of diffusible substances. From one diffusion is relatively rapid and com-

pleted in 15 to 30 minutes. In the other, which constitutes the major fraction, diffusion occurs at a rate indicating a diffusion coefficient about 1/300 that in aqueous solution.

METHODS

General. The sciatic nerves of Rana pipiens and R. catesbiana were used exclusively. The experiments were concerned with the kinetics of entry and exit, chiefly the latter because of the greater precision possible. Thus, a single nerve or set of nerves can supply complete data on ionic exchange or diffusion as a function of time; this is possible with the technique previously described (Shanes, '50a, '51a), whereby small quantities of medium, after equilibration of the nerves in appropriate solutions, are placed successively in contact with the tissues and then completely removed for analysis. The nerves are analyzed at the end of each series, thereby providing a check on the rates indicated by the media; moreover, addition of the figures from the media and nerves permits determination of the initial nerve content as well as reconstruction of the curve of "average desaturation" for comparison with the theoretical curve derived for a homogeneous cylinder (Hill, '28).

The entire length of Rana pipiens nerve, from the plexus at the cord to the entry of the peroneal and tibial branches at the knee, was employed. However, in the majority of experiments, particularly with radioactive agents, only the short, uniform, unbranched segment of the R. catesbiana sciatic proximal to the origin of the tibialis and peroneus was used. The nerves were loosely tied to glass rods, with which they were inserted into the 4 pyrex circulation units of the type employed in 1950. In conjunction with suitable reservoir units, small volumes of solution (ca. 1 ml) could be placed in contact with the nerves, agitated continually by a stream of O_2 bubbles, and replaced at appropriate intervals with fresh solution. Care was taken to see that the nerves did not make contact with surfaces. Except for the beginning portion of each experiment, the collecting solutions were left in contact

with the nerves for intervals which increased by a factor of 2. The final level of the particular diffusing agent being studied was about the same in each collection. Under these conditions the effect of the small rise in concentration in the medium on the diffusion rates was negligible. Thus, the greatest error occurred when three nerves — amounting to 100 mg — were used. In the case of diffusion of 1/3 potassium Ringer's from the nerves into Ringer's, the external potassium concentration of 1.7 μM/ml changed 10–20%, which represents a reduction in the initial concentration difference, between extracellular spaces and medium, of only 1%. In the corresponding case for Cl^{36} the count rate of the fluid in the extracellular space after equilibration was 140,000 counts/min. ml,[1] whereas the collected media samples seldom exceeded 1000 counts/min. ml, an error in the concentration difference of less than 1%. For the slower diffusing sucrose this factor was smaller. In the majority of experiments, where single uniform segments weighed 1/5 or less than in the above, the error was proportionately less still.

Companion nerves frequently were analyzed before and after the equilibration times corresponding to the above "media experiments." Data so obtained provided a check on the latter. Tissue always was weighed before and after each new procedure. Where weight changes were small, the mean weight usually was employed; otherwise the initial weight served as the reference. Experimental procedures which caused appreciable weight changes were avoided in other than exploratory work since the significance of emission curves obtained under these conditions was uncertain. Thus, Ringer's in which all the sodium is replaced by potassium, causes a substantial increase in weight (table 2). Desheathing causes a 40% increase in weight (Shanes, '51b and '53) which is half complete within 20 minutes and is progressive thereafter; because of the instability of such preparations our research was restricted to intact nerve trunks.

[1] Counts obtained with the flow counters, in which efficiency is 50% or better (see below).

For the studies of the exit of experimental agents, the nerves usually were equilibrated for 16 hours in oxygenated solutions containing the diffusible agent in suitable concentration. This procedure provides a relatively stable preparation with respect to weight, potassium and sodium (Shanes, '52). Data are expressed relative to the weight at this time. Variability of the data is given as the standard error for small samples. Solutions were maintained at a pH of 7.2 with a small quantity of phosphate buffer and, unless otherwise indicated, were iso-osmotic with Ringer's made up as previously described (Shanes, '52).

Potassium and sodium. Analyses were carried out with the Beckman no. 10300 flame spectrophotometer (see Shanes, '50a, '51a, '52). In earlier experiments two paired sets of nerves, each set composed of three nerves tied together at their ends, contributed to a single day's run. The use of more than one nerve per unit lowers exchange rates (fig. 1 B), consequently in most later experiments single nerves were employed. Also, it was noted that thread does not completely give up the experimental agents during brief washing and otherwise distorts the emission curve (fig. 1 B); therefore, in most routine experiments it was replaced with 3 mil surgical tantalum wire for tying purposes.

The sodium and potassium content of the tissue was determined by analysis of the aqueous extract of the ash obtained after 16 hours' incineration at 500°C.; the nerves were first blotted on filter paper, their ends cut off, and then dried to constant weight.

Radioactive agents. Long life radioactive chloride [2] (Cl^{36}) and C^{14} labeled sucrose [3] were employed with the same experimental techniques used for potassium. Two milliliters of the original solution containing Cl^{36} were neutralized and

[2] Supplied by the Oak Ridge National Laboratory as approximately 1N hydrochloric acid with an activity of 5 μc/ml. Radiochemical purity was checked by running an absorption curve.

[3] Supplied by the Nuclear Instrument and Chemical Corp. with a purity estimated at better than 99% and an activity of 0.5 μc/mg. Radiochemical purity was checked by running an absorption curve.

made up to an isotonic 50 ml radioactive Ringer's estimated to have an activity of ca. 0.2 μC/ml. Radioactive sucrose usually was added to Ringer's or to Cl^{36} Ringer's to the extent of 2 mg/ml (1 μC/ml), thereby altering the osmotic strength less than 3%. Thus, the activities involved were on the tracer level, and the sucrose concentration used was extremely low.

The reproducibility of emission curves was found to be dependent on the procedure used in transferring the nerves from the preliminary soaking solutions to the circulation units. The technique adopted included blotting the wires, nerve and supporting glass rod with filter paper first, then giving a quick preliminary dip in inactive solution, and finally mounting in the circulation unit. Failure to use both parts of this routine was prone to give an excessive initial release and excessively high total emission compared to that estimated from analyses of companion nerves, presumably because of contamination.

The escape of radioactive agents to regular Ringer's was followed by repeated collections of small volumes in contact with the nerves. Duplicate media samples, of 0.3 ml each, were deposited on stainless steel planchets, with "Roccal" and small discs of lens paper to achieve uniform spreading; these were carefully heated to dryness under infra-red lamps.[4] Counting usually was done with two gas flow counters of the type described by Robinson ('50) which, with P-10 gas [5] to give proportional counting and with the discriminator on our scalers [6] appropriately adjusted, gave uniform backgrounds of 6 counts/min. All experimental counts were at least tenfold higher than background and were carried usually to 4000 or greater, hence the standard deviation of individual counts was of the order of 1.5%. Duplicates were run on separate counters and one standard reference was run on both units at the beginning and end of the day as well as on successive days as a check on systematic differences and on drift. The

[4] We are indebted to Mr. Dean Cowie for suggesting this procedure of deposition.

[5] Methane-argon supplied by the Matheson Co., Inc.

[6] Manufactured by the Nuclear Instrument and Chemical Corp.

"hot" solutions in which the tissues had been soaked were diluted in the same medium used for following emission as a further check; thus a reference was always available to adjust the counts found in the media to a relative scale. Although agreement between duplicates was usually within 5%, differences up to 10% generally were accepted and averaged.

The activity of nerves, after the ends had been removed, was determined by soaking the tissue in 0.8 ml distilled water under conditions of intense vibration — for two hours for Cl^{36} and 4 hours for labeled sucrose — and then depositing

TABLE 1

Comparison of the Cl^{36} and C^{14} labeled sucrose (S), in counts per minute, estimated for planchets containing both, using (A) an end window Geiger counter with and without absorber and (B) an end window counter without absorber and a gas flow counter. Counts given are those for a Geiger tube without absorber.

PLANCHET	A		B	
	Cl^{36}	S	Cl^{36}	S
1	200	108	195	113
2	168	103	161	110
3	128	79	122	85
4	158	114	158	114
5	207	143	195	155
6	734	579	750	580
7	723	522	707	539
8	166	59	163	62

0.2 ml of the resultant solutions on planchets as above. This procedure was considered satisfactory since (a) additional soaking in water did not elevate the count further and (b) the chloride space computed from data so obtained is in close agreement with that found by Fenn et al. ('34) with conventional procedures. Allowance was made for the contribution of the water content of the nerves to the total volume.

For certain experiments it was desirable to determine Cl^{36} and sucrose simultaneously in the nerves or in emission experiments. For comparison of chloride and sucrose space this was particularly necessary to minimize statistical variation.

Two methods were found satisfactory: (1) Counts were run with a thin window (1.6 mg/cm^2) Geiger tube (Tracerlab TGC2) with and without two layers of "Reynolds Wrap" aluminum foil as absorber; a complete Tracerlab automatic counting unit was available which made possible round-the-clock counting. The background was 18 counts/min. with a maximum range of variability of only 1 count/min. Thus, data for nerves could be collected in a reasonable time and with good precision. (2) For media, count levels were too low for use of the Geiger tube with an absorber; however, comparison of the counts obtained with the Geiger tube alone with that in the flow counters gave sufficient discrimination to permit satisfactory measurement of both Cl^{36} and sucrose in the same samples. The agreement obtained with both methods on the same planchets is shown in table 1. The relative transmission factors for chloride and sucrose were approximately 90 and 10%, respectively, for the first procedure, and 30 and 10% for the second.

Unless indicated otherwise, experiments were carried out in a constant temperature room at 25 $\pm$ 1°C. Low temperature studies were conducted in a cold room at 4°C. Nerves were placed in the equilibration solution at cold room temperature one to one and one-half hours prior to the emission run, after the usual long equilibration at higher temperature as in the controls. All apparatus, gas and solutions used at low temperature were left in the cold room for at least 16 hours previous.

RESULTS

Nerve analyses

Sodium-potassium. The first attempt at measuring sodium-potassium exchange was by immersion of nerves in a Ringer's with all the sodium replaced by potassium. Potassium phosphates were employed for the buffer and calcium was present as usual. The emission of sodium into this medium was followed (see below) and the nerves were analyzed after 13 hours' exposure. Table 2 summarizes the nerve data.

TABLE 2

Weight changes and potassium and sodium contents of 16 hour, Ringer's-equilibrated nerves, following an additional 13 hours' exposure to 100% potassium Ringer's.[1] *Earlier figures for potassium and sodium contents of 16 hour equilibrated tissue, and following an additional 12 hours in Ringer's (Shanes, '52), are given for comparison. R. pipiens. 25°C.*

PRETREATMENT	WEIGHT CHANGE	POTASSIUM CONTENT		SODIUM CONTENT	
	(%)	*μM/gm wet wt.*[2]	*μM/gm dry wt.*	*μM/gm wet wt.*[2]	*μM/gm dry wt.*
Equilibration and potassium	33.0 ± 2.9	88.2 ± 2.7	560 ± 47	2.64 ± 0.54	16.3 ± 3.0
No.	6	12	12	12	12
16 Hr. equilibration		33.7 ± 1.7	156 ± 8.0	70.1 ± 1.8	328 ± 20
28 Hr. equilibration	0	33.6 ± 1.8	162 ± 6.0	78.5 ± 2.4	382 ± 25

[1] All sodium replaced with potassium.

[2] Per gram *final* wet weight.

The potassium Ringer's causes a 33% gain in weight. The figures in table 2 demonstrate that the potassium increment (per gram dry weight) is equal to the sodium lost plus (1) a potassium gain (36 μM) equivalent to that in potassium Ringer's entering as the source of the weight increase and (2) an additional uptake (54 μM) of extracellular cation which occurs during 12 hours following equilibration, as may be seen by comparing the sodium contents of 12 and 28 hour equilibrated nerves. Thus, on a dry weight basis, the sodium loss in potassium Ringer's is 312 μM/gm; the potassium gain is 404 μM/gm, of which 90 is due to other than exchange, as pointed out above, hence 314 is the figure to be compared with the sodium loss (312 μM/gm). The agreement, therefore, is good.

Although sodium-potassium exchange observed in such experiments (see below) might be useful for the establishment of the order of magnitude of the rates involved, the large water uptake precluded its use for precise determinations of the exchange coefficient.[7] Less drastic conditions therefore were sought.

A series of experiments was carried out to determine whether sodium lack is by itself responsible for the water uptake. The results (table 3) show not only that the absence of sodium fails to cause the large weight gain but prevents the weight increase which normally occurs during 16 hours' equilibration in Ringer's. However, the low sodium Ringer's is by no means innocuous, for, as pointed out earlier (Shanes, '51b, '52) and as may be seen in table 3, a significant although small (15%) potassium loss occurs at low sodium levels. The exposure to Ringer's for 7 hours restores the normal sodium level, presumably in the extracellular space.

An additional set of experiments, summarized in table 4,[8] demonstrated that an elevated potassium concentration does not cause weight changes any different from sodium when

[7] This phrase is preferred to "diffusion coefficient" when ionic exchange is involved.

[8] A preliminary report of these results was given earlier (Shanes, '48b).

the latter ion is present at normal levels. Thus, when the tonicity of Ringer's is doubled by the addition of KCl or NaCl, the maximal shrinkage of nerve, which occurs in about one and one-half hours (Shanes, '48a), is the same in either case, and the weight gain over a subsequent 20 hour period is also the same. The functional properties of these nerves apparently were not affected adversely, for return to normal Ringer's caused a percentage change in weight equal to that

TABLE 3

Weight changes during 16 hours' equilibration in Ringer's and in a 2 mM/l sodium Ringer's,[1] *and the sodium and potassium contents of the nerves after an additional 7 hours in normal Ringer's solution. R. pipiens.*

NORMAL RINGER'S				
Weight change	Na	K	Na	K
%	*μM/gm wet wt.*		*μM/gm dry wt.*	
4.7 ± 0.7	69.9 ± 1.4	35.5 ± 1.0	319 ± 9.6	171 ± 5.4
No. 22	6		6	
LOW SODIUM RINGER'S				
Weight change	Na	K	Na	K
%	*μM/gm wet wt.*		*μM/gm dry wt.*	
0.18 ± 0.64	69.0 ± 2.1	30.2 ± 0.9	331 ± 9.3	151 ± 2.6
No. 16	6		6	

[1] Choline or sucrose replacing the sodium.

TABLE 4

Weights, in per cent of initial weight in Ringer's, of R. pipiens nerve and muscle after exposure to 2 × hypertonic solutions in which the indicated substance was added to the medium in amounts osmotically equivalent to 0.11 M NaCl according to freezing point data. Parenthetical figures give the number of nerves providing the means.

TIME	SCIATIC NERVE				
	Control	Glucose	Sucrose	NaCl	KCl
hr.					
1.5	101±0.4(4)	78.9±1.3(8)	78.6±0.6(6)	84.2±1.1(7)	86.0±2.2(4)
20	107±1.9		86.8±0.8	94.2±1.7	95.8±2.6
20.5	Return to Ringer's →				
22	104±3.2		107±1.9	110±1.0	112±1.8
	SARTORIUS MUSCLE				
3		75.8±1.1(8)	79.8±2.0(4)	75.9±0.9(4)	

obtained during the first one and one-half hours in hypertonic solution, and good action potentials were soon found even in the nerves which had been treated with potassium.

The data of table 4 lead to an additional important conclusion: Unlike the situation in frog muscle (Boyle and Conway, '41) and crab nerve (Shanes, '46), anion permeability is extremely low in frog nerve fibers under ordinary circumstances. As a result, KCl cannot penetrate as such to give rise to water uptake when it replaces NaCl in the medium.

The osmotic and low sodium experiments suggested that replacement of a sufficiently small quantity of sodium with potassium would enable the nerves to maintain normal weights and thereby to serve as preparations suitable for the study of sodium-potassium exchange. This was found to be the case when potassium was substituted for 1/3 of the sodium (table 5). Nerves soaked for two and 6 hours in such potassium Ringer's are seen to exchange equivalent amounts of sodium and potassium when their contents are compared with a suitable control. The exchange by two hours is approximately 80% of that at 6 hours. The excess potassium space, i.e., the percentage of the nerve occupied by the solution with the added potassium, is found to be 54 and 65 at two and 6 hours. This is of the magnitude found by Fenn et al. ('34) to be the chloride space, which these authors demonstrated by two clear-cut procedures to be largely extrafibrillar. Hence, the fibers probably were not penetrated by a significant amount of the excess potassium.[9]

[9] Since the validity of this conclusion is important in an evaluation of the site of action of excess potassium, note must be made of two statements to the contrary in the literature. The data on which they are based can be shown to be consistent with the above. Thus, Fenn et al. ('34) found that at isotonic potassium levels the entry of potassium is greater than can be accounted for in terms of the extracellular space; however, their data are in accord with those in table 2, where the large uptake is seen to be a consequence of the change in nerve volume. Feng et al. ('50) observed that an isotonic (474 mg %) potassium Ringer's increased the potassium content of desheathed toad's nerve from 200 mg % to 420–460 mg % in 40 minutes. On the basis of the *total* final quantity in the nerves, they conclude that some of the excess potassium entered the fibers; however, the initial content must be deducted from the total to evaluate the *increment* (220–260 mg %), from which the excess potassium space is calculated to be no more than 55%, a figure also in keeping with the above.

A discrepancy is apparent in the relative amounts of sodium and potassium transferred after 16 hours in potassium Ringer's in the absence of a previous 16 hour equilibration in Ringer's. It will be recalled (table 2), and it is apparent in

TABLE 5

Weight changes and final sodium and potassium contents of nerves under various conditions of treatment with and without excess potassium in the medium. R. pipiens

COLUMN	A	B	C	D	E	F
Time in Ringer (hr.)	0	16	16	16	0	0–12[1]
Time in 1/3 K Ringer (hr.)	0	0	2	6	16	16
Weight change in K (%)	..	4.7±0.7[2]	0.75±0.6	0.87±1.3	6.3±0.7	..
Na (μM/gm final wet wt.)	61.4±1.8	74.2±1.4	55.8±2.0	50.8±3.1	45.1±1.5	73.2±2.1
K (μM/gm final wet wt.)	40.4±0.3	37.2±1.2	57.5±2.4	62.1±1.4	59.1±0.9	43.3±1.6
No.	6	9	6	6	6	16
— B (Na)			19.6	23.5	29.1	
— B (K)			20.3	24.9	21.9	
— F (Na)					28.1	
— F (K)					15.8	
— A (Na)					16.3	
— A (K)					18.7	
Excess K space (%)[3]			54	65		

[1] Nerves immediately immersed in 1/3 K Ringer's, then leached for 12 hours in normal Ringer's.

[2] Mean based on 20 measurements for 16 hours in Ringer's. A previous value based on 60 determinations was 7% (Shanes, '52).

[3] Obtained by dividing the mean sodium and potassium changes by the potassium content of the potassium Ringer's (37 μM/ml).

table 5, that the nerves tend to gain sodium with either no or a slight loss in potassium. The problem therefore arises as to the appropriate control for determining the net change. The results of using different possible controls — fresh nerve (column A), 16 hour, Ringer's equilibrated nerve (column B), and potassium soaked nerves after 12 hours' washing in Ringer's (column F) — give either too low ionic exchange compared to the two and 6 hour results, or discordant values for potassium and sodium (table 5).

The inadequacy of available controls is suspected as the source of discrepancy. On this basis the average of the sodium and potassium changes obtained with a particular control should still give the correct amount of exchange. In keeping with this it can be seen that the long equilibrated controls give averages practically equal to the 6 hour figures. In any case it is clear, from any of the controls, that the potassium uptake does not exceed significantly that which would be contained in the chloride space.

C^{14} labeled sucrose and Cl^{36}. All measurements were carried out with both radioactive agents present so that they were individually determined on the same nerves as outlined under "Methods." The relative uptake, referred to as "space," is shown for R. pipiens and R. catesbiana in table 6. The figures for chloride at the later times are slightly larger than those reported by Fenn et al. ('34); this difference is partly ascribable to the uptake of Ringer's as the source of weight gain during 16 hour equilibration. In both species the sucrose space is significantly smaller than the chloride space; in R. catesbiana, this difference is seen to be independent of time, and therefore probably is a genuine difference in the nerve volume available to chloride and sucrose. That different diffusion rates are not responsible is shown by the kinetics of diffusion described below. Thus, by 16 hours even sucrose is probably within better than 5% of equilibrium.

A difference in sucrose and chloride space was predicted earlier (Shanes, '48b) on the basis of the greater osmotic effectiveness of sugars, now shown in detail in table 4. This

effect is not obtained in muscle. The gradual return towards the original weight over the following 20 hours is seen to be the same whether electrolytes or sugars render the Ringer's hypertonic; moreover, this weight gain is only slightly larger than that of the controls, and therefore not ascribable chiefly to the hypertonicity or to the particular substance added in excess. It may represent in part an enlargement of the extracellular space such as occurs with dramatic rapidity upon removal of the sheath. In keeping with this, such preparations gain sodium without a corresponding loss in potassium

TABLE 6

The chloride and sucrose (S) spaces of frog nerve estimated as the percentage ratio of nerve activity/gm to activity/ml of soaking solution. The ratio of the chloride to sucrose space is given with the standard error based on the two spaces as estimated in the same nerves.

SPECIES	EXPOSURE TIME	Cl^{36} SPACE	S SPACE	$\frac{Cl}{S}$
	hr.	%	%	
R. pipiens	16	65.1 ± 1.5	43.9 ± 4.3	1.67 ± 0.24
	No.	9	9	9
R. catesbiana	5	52.5 ± 1.7	38.6 ± 2.1	1.39 ± 0.10
	No.	6	6	6
	23	61.8 ± 1.0	47.2 ± 1.5	1.32 ± 0.05
	No.	6	6	6

(cf. columns A and B in table 5 after correction for the weight gain of 16 hour preparations).

Since the penetration by sucrose and chloride is not complete by one and one-half hours (70 and 90% of completion, respectively, according to fig. 3 A), shrinkage by this time in an excess of these agents should exceed that at equilibrium if water diffusibility is not a limiting factor, particularly in the case of sucrose. However, the somewhat greater return to the original weight, to be expected in sucrose, is not apparent in table 4.

Further evidence of the absence of toxic effects by 1/3 potassium Ringer's was seen in the absence of any demonstrable

action by such solutions on the chloride space in R. pipiens. Thus, in 6 paired nerves, the Cl^{36} space of controls, after 16 hours of equilibration, averaged $59 \pm 1.9\%$, whereas the mates exposed to the high potassium concentration for the same period had a chloride space of $62 \pm 1.1\%$.

Media analyses

Sodium-potassium. The precision of measurement of a particular agent appearing in the medium as a result of its loss from nerve is in proportion to its amount in the medium initially. The complete absence of sodium in the potassium Ringer's first employed promised maximum sensitivity; however, as already pointed out, a substantial weight gain occurs during the course of such experiments and therefore the exchange observed can be considered merely indicative of the order of magnitude of the rates involved.

The sodium emission curve obtained is given in figure 1 A. Since the semi-log plot is not linear, we are not dealing with an exponential process and the concept of "time constant" is not applicable as a measure of basic rate. We shall therefore merely refer to e-time (i.e., the time required for the changes to reach 1/e or nearly 1/3 of completion) without any assumption as to the time course of the process. Thus, the e-time in this series was approximately 90 minutes.

It must be stressed that the above measurements were obtained with three nerves per unit and with thread, washed briefly in Ringer's prior to each run, for tying purposes. Both procedures affect the emission curves (fig. 1 B). The former is of interest since the release of potassium from the fibers under a variety of conditions (Shanes, '51b, '52) was studied with more than one nerve per unit, hence the kinetics of diffusion when nerves are tied together is useful for mathematical analyses of the earlier data. The retentive property of thread, however, introduces an artifact which is not pertinent either to the present or earlier experiments; therefore, thread ligatures were avoided in most future work.

The limitations of sensitivity required the use of groups of three nerves in the study of the emission of potassium to Ringer's following equilibration in a 1/3 potassium Ringer's. The results are summarized in figure 2 A. Because the exact excess of potassium taken up by the nerves (i.e., the initial content used to construct the "desaturation" curves) was uncertain owing to the question of appropriate control levels, the net losses are plotted relative to that obtained in 240

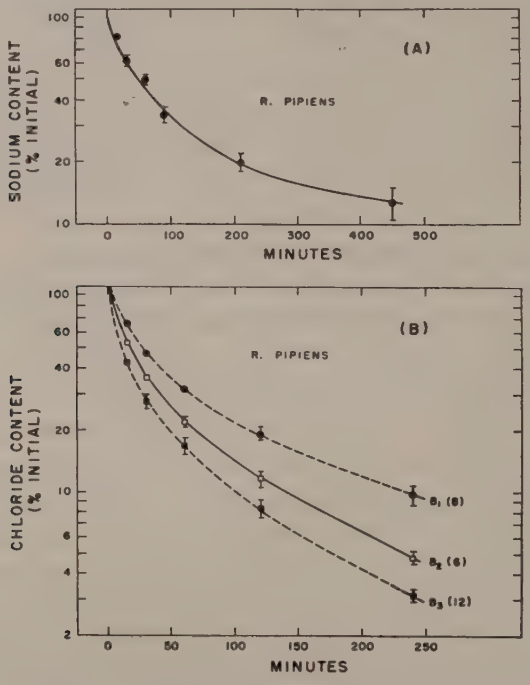


Fig. 1 (A) Decline of the sodium content of frog nerve in a Ringer's with all sodium replaced by potassium. (B) Decline of the Cl^{36} content, in inactive Ringer's, of nerves previously equilibrated for 16 hours in active solution: B_1 nerves run in groups of three tied together at their ends with thread. B_2 as in B_1, but tantalum wire used instead of thread. B_3 single nerves suspended in solution by tantalum wire tied to the ends. Parenthesized figures in this and subsequent graphs give the number of runs and hence number of data for each point. Vertical lines express $\pm$ the standard error; where these are absent the standard error is equal to or less than the dimensions of the symbols. The initial amount is the sum of the losses determined from the collected samples and of the amount found in the nerves at the termination of the experiments. 25°C.

minutes. If 26 μM/gm wet weight is used as the total uptake during equilibration, the desaturation curve obtained has the following characteristics: An initial rapid phase, ending by 30 minutes, is followed by an exponential decline with a time constant of 115 minutes. This is comparable to the time constant of 140 ± 45 minutes (Shanes, '52) obtained for the diffusion of sodium as the chloride into sucrose.

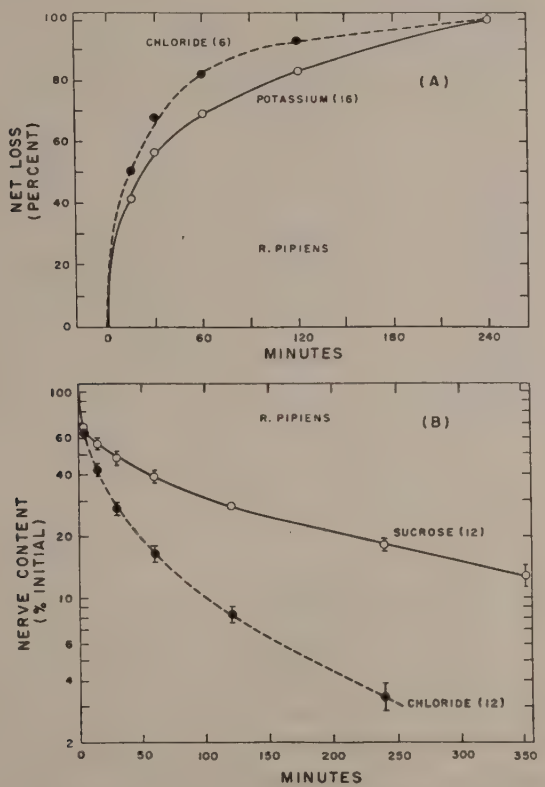


Fig. 2 (A) The net loss of potassium to Ringer's, from groups of three nerves tied together with tantalum and previously exposed for 16 hours to 1/3 potassium Ringer's, compared with the corresponding curve for Cl^{36}. The total loss by 240 minutes (24.3 ± 1 μM/gm wet weight for potassium) is taken as 100%, and earlier values are given relative to this. (B) Comparison of the decline of the radioactive sucrose and chloride contents of single nerves in inactive Ringer's following equilibration for 16 hours in "hot" Ringer's. 25°C.

Radioactive chloride and sucrose: R. pipiens. The effect of different experimental conditions on chloride exchange is shown in figure 1 B. The lack of linearity in all curves demonstrates that the exchange does not occur as a simple exponential process. In addition, the figure demonstrates that tying several nerves together by their ends, and the use of thread instead of tantalum for ligation, both reduce the exchange rate. The e-times for curves B_1, B_2 and B_3 are 50, 30 and 15 minutes, respectively.

In figure 2 A the liberation of Cl^{36} by exchange is compared with the exchange of potassium for sodium under similar conditions, viz., from groups of three nerves tied together with tantalum. The former occurs with greater rapidity.

Chloride exchange is compared with the diffusion of sucrose in figure 2 B. These and subsequent curves were obtained under conditions considered to approach the ideal, e.g., with single nerves and tantalum ligatures. The sugar was selected for comparison since it is unlikely to penetrate the fibers and unlikely to be metabolized. Like chloride, but to a more marked degree, it emerges rapidly at first and then slowly. The fast and slow components of sucrose emission probably cannot be attributed to the extracellular and intracellular spaces, respectively. Rather, it seems necessary to seek for the cause of the difference within the extracellular space itself.

Additional experiments were undertaken under still simpler conditions to rule out other possible extraneous factors. Thus, the different diameters of the sciatic and its branches may have contributed to the change in rate with time. Consequently, subsequent series were carried out on an extremely limited segment of R. catesbiana sciatic — the unbranched uniform segment, approximately 1.1 mm in diameter, immediately proximal to the origin of the *tibialis* and *peroneus* nerves. The results so obtained will now be described.

Radioactive chloride and sucrose: R. catesbiana. The data for sucrose and chloride obtained at 25° and 4°C. are summarized in figure 3. At low temperature a rapid initial re-

lease, distinct from the rest of the emission curve, is particularly evident. Except for sucrose at low temperature and later times, the curves again show no perfectly linear semilogarithmic decline. The e-times for sucrose and chloride are

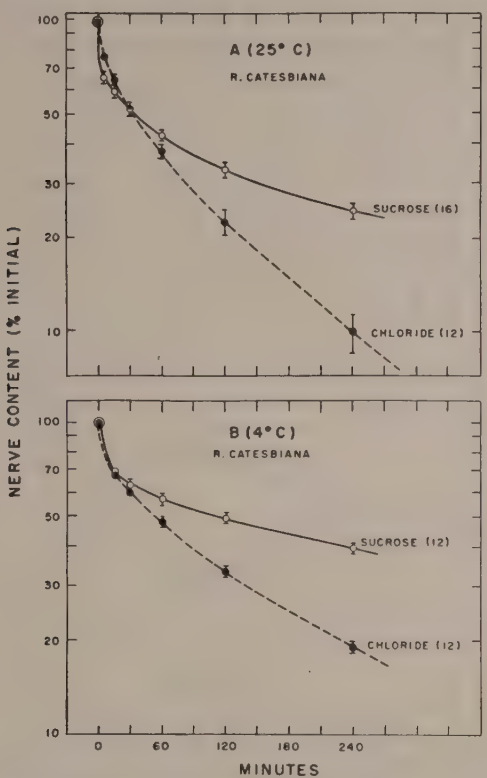


Fig. 3 Comparison of the decline, in inactive solution, of radioactive sucrose and chloride in single bullfrog sciatics at 4 and 25°C. As usual, equilibration in the isotopes was for 16 hours at 25°C.; an additional hour or hour and a half of equilibration at low and high temperature preceded the experiments.

100 and 65 minutes, respectively, at 25°C., and 200 and 90 minutes at 4°C.

The possibility was examined that the fast component is a consequence of deterioration of the nerves during the long initial soaking period. This was done by soaking fresh nerves for only 4 hours in radioactive solutions, following emission as usual, and then soaking the same nerves for the customary

16 hours and comparing the loss. The results based on 8 such experiments are shown for chloride and sucrose in figure 4. Since nerve data were not available following the short experiment, comparison is made on the basis of normalized curves. Thus, the amount lost by 60 minutes is taken as the reference level and arbitrarily referred to as 1. On this basis the initial rapid release is even more evident for the fresh

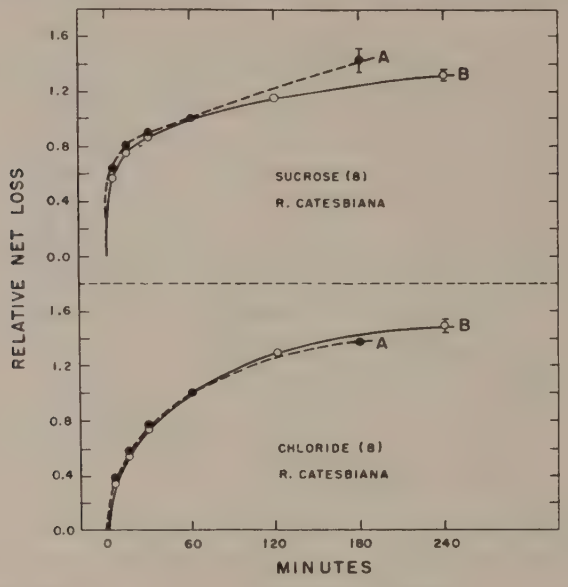


Fig. 4 Comparison of the loss of radioactive chloride and sucrose to inactive Ringer's from A, fresh nerves equilibrated in the active media for 4 hours and, B, the same nerves, 24 hours old, equilibrated for the usual 16 hours in "hot" solution. All data given relative to the total loss by 60 minutes. 25°C.

nerve than for those which had aged. This does not mean, of course, that more had come out initially; actually the absolute amount was somewhat smaller, for the net amount which had appeared by 60 minutes was less in the nerves exposed only 4 hours to radioactive solutions — a natural consequence of the smaller amount of radioactive material which could be taken up in the shorter time. Thus, the chloride and sucrose released in 60 minutes by these nerves were 75 and 70%, respectively, of that released after a 16 hour soaking period.

One series of experiments was carried out in an effort to determine whether the ends of the nerves (10–15% of the total length of nerve) were the source of the rapid release. Nerves were exposed to radioactive sucrose solutions for 5, 15, and 60 minutes, the ends cut off, and the remainder extracted for activity measurements. Since the "saturation"

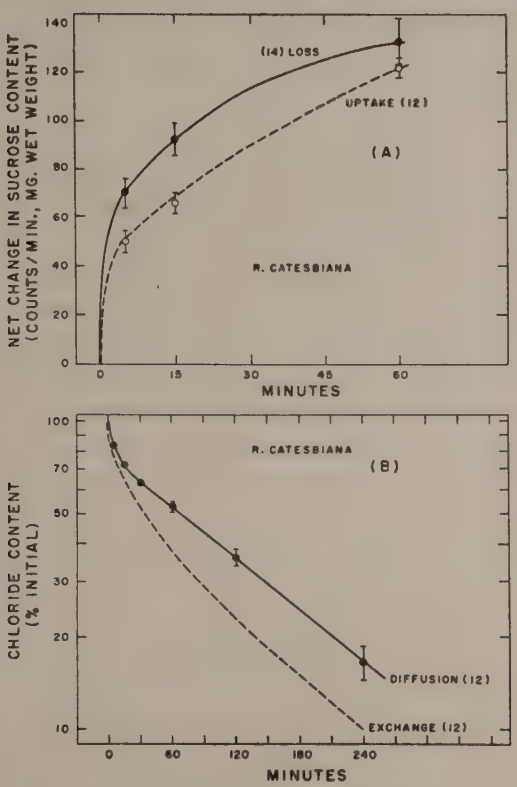


Fig. 5 (A) Comparison of the net loss of radioactive sucrose to inactive Ringer's from nerves equilibrated for 16 hours in "hot" Ringer's, with the uptake in the same radioactive solution by nerves equilibrated previously for 16 hours in normal Ringer's. Uptake estimated by direct measurements on the central segments of the nerves, losses by the usual analysis of collected samples. (B) Decline in the radioactive chloride content of single bullfrog sciatics in a Ringer's with all the NaCl replaced by sucrose of equivalent osmotic strength (7.1%). Since this is accompanied by the additional loss of sodium, rather than by exchange with chloride in the medium, it is referred to as diffusion. The exchange curve from figure 3 A is given for comparison. 25°C.

or uptake curve must mirror the "desaturation" or release curve as long as purely passive phenomena are involved, any deviation may be attributed to the ends. In figure 5 A the absolute amount of activity taken up is compared with that released in earlier experiments. At 60 minutes the amounts approach each other, but at the shorter times they are consistently less. Nevertheless it is quite clear that the nerve trunk proper is responsible for at least a major proportion (70%) of the rapid uptake (and hence release) of sucrose during the first 5 and 15 minutes.

A final series was conducted to compare chloride diffusion, as NaCl, with exchange. For this purpose Cl^{36} emission, after the usual 16 hour equilibration in radioactive Ringer's, was observed in a Ringer's in which all the NaCl was replaced with sucrose; some electrolyte (sodium phosphate buffer, KCl, and $CaCl_2$, in the usual concentrations) was present in the latter, but contributed to only 4% of the tonicity. The results of 12 runs are summarized and compared with the exchange curve in figure 5 B. The rate of diffusion is substantially lower and follows an exponential time course after the first 15 minutes. Its exponential nature is consistent with the findings of Fenn et al. ('34) with ordinary chemical methods (see Shanes, '51b, '52). The initial rapid loss is particularly clear in the diffusion curve.

DISCUSSION

These results, in the light of available data, indicate (1) that the exchanges of excess potassium for sodium and of radioactive chloride for inactive chloride are largely extracellular phenomena, (2) that the extracellular space is composed of at least two distinct phases — a smaller one in which exchange and diffusion occur with relative rapidity, and a larger one which meets considerably more resistance, and (3) that a third phase exists which is accessible to potassium and chloride but not to sucrose.

With regard to the last situation, the possibility of entry of the ions into the fibers appears unlikely in view of the fail-

ure of the nerves to swell when potassium replaces sodium — a swelling which occurs in systems permeable to both chloride and potassium (Boyle and Conway, '41; Shanes, '46, '50b). Moreover, Fenn et al. ('34) describe two experimental procedures which lead to the conclusion that all the nerve chloride is extracellular. Another possibility is that myelin can take up ions but not sugars. The cross-sectional areas of myelin and axoplasm are about 1 to 1 (Renyi, '29); if a major portion of the myelin were available to ions, the non-sucrose space [10] would be to the non-chloride space [11] as 2:1. When the dry weight of 22% (Shanes, '52) as well as the chloride and sucrose spaces are taken into account, the ratio for R. pipiens is 34:13 and for R. catesbiana 31:16, which appear to be of the correct order of magnitude. More remote sheaths cannot be considered responsible for the selectivity since glucose, which exerts similar osmotic effects to sucrose, is utilized under anaerobic conditions (Shanes, '52) and hence must be able to reach the fibers at least at the nodes. More direct data are required on the histology of the frog nerve trunk and the electrical characteristics of specific structural elements.

The rapid component of the emission curves appears attributable to material contained in the aqueous phase of the nerve sheath (epineurium). This is in accord with the observation that relatively small concentration gradients may lead to the rapid development of effects in intact nerve (Lorente de Nó, '50). Slow emission therefore would appear to be from the extracellular space in the fiber-containing core of the trunk.

An estimate of the relative dimensions of the two phases is in accord with this viewpoint. Consider, for example, the situation in bullfrog nerve. In this case the sheath thickness may be taken as 40 μ, a figure indicated by histological sections (Lorente de Nó, '50). Thus, in a 1.1 mm trunk, the core diameter is 1 mm. If, as for whole nerve (Shanes, '52), 80% of the

[10] The space unoccupied by sucrose.

[11] The space unoccupied by chloride.

sheath weight is available to dissolved substances, the contribution to the extracellular space is 0.11 ml/gm wet weight of nerve. Since 0.62 and 0.47 ml/gm are the total chloride and sucrose space, respectively (table 6), then the phase of rapid release would be 18% of the total for chloride and 23% for sucrose.

Inspection of figure 5 B, where the initial component for chloride is most distinct, indicates a rapid phase of 20%, and figure 3 B, for sucrose, 35%. If these are reduced to 70%, as suggested by direct nerve analyses (fig. 5 A), they become 14 and 23%. This agreement with the predicted percentages is good, probably better than is to be expected in view of the uncertainty involved in the extrapolations to estimate the magnitude of the rapid component.[12]

As a working hypothesis, therefore, the sheath and core will be considered to constitute the source of the rapid and slow components of emission. A first approximation may now be made of the diffusion coefficient for the sheath. The epineurium will be assumed to be an infinite homogeneous plane sheet in contact at the inner surface with an impermeable wall; the result is divided by 2 to allow for a final steady state gradient which limits the sheath loss to half the original content. On this basis, if the "average desaturation" is 95% complete in 15 minutes, the coefficient can be shown to be (Hill, '28) 10^{-6} cm^2 $min.^{-1}$.

Although this is no more than a rough estimate, it suffices to show that the diffusion is slow compared to that in free solution (table 9). Indeed, it is of the order obtained for the fiber-containing core when the major diffusion resistance is considered to reside in the core (see below); this identity is not accidental for, as will be shown in a subsequent report, these sheaths, containing only radioactive Ringer's, can account for and do duplicate the kinetics of whole nerve. The computation demonstrates that the diffusion constant may be

[12] It should be noted that a curve fitting procedure, employed below to estimate a diffusion constant for the slow phase, gives 10% and 25% for the rapid components of chloride and sucrose.

low in the sheath although the entry or exit of substances is relatively rapid. This follows from the high surface to volume ratio (1/thickness = 20 mm^{-1}) characteristic of a thin peripheral structure such as the epineurium. In the case of the 1 mm core, the ratio equals 2/radius or 4 mm^{-1}, or is 1/5 that of the sheath. This would contribute to the difference in rates in these two phases of the extracellular space.

If the diffusion coefficients were identical in the epineurium and core, the solution for a homogeneous cylinder, as given by Hill ('28), should be adequate to describe the total diffusion process. That such is not the case can be shown in the following way. From Hill's curve of "average saturation" (fig. 5 in Hill, '28) one can read off a time dependent variable, kt/r_o^2, as a function of the net loss. The ratio k/r_o^2 is that of the diffusion coefficient to the square of the cylinder radius, and therefore is a constant. Hence if the values of kt/r_o^2, taken from the theoretical curve for different net losses, are divided by the corresponding times (t), conformance to the theory of diffusion for a homogeneous cylinder will be indicated by the constancy of the quotient with time. In tables 7 and 8, in the columns designated as 0 correction factor, may be seen the continual decline of the quotient with increasing time for both sucrose and chloride emission at 4 and 25°C.

We may now inquire whether the proposal of two distinct phases will account for the deviation from simple theory. If so, it might be possible to deduct the contribution by the sheath, which appears to be completed early, from the total and thereby to reconstruct the emission curve for the slower phase. When the proper correction is made, k/r_o^2 should become constant with time as required and, since r_o is known, this ratio will then provide an estimate of k for the fiber-containing core. Such constancy is approached when the correction factor is 25% for sucrose and between 5 and 10% for chloride, as may be seen in tables 7 and 8. If the values 25 and 10%, respectively, are employed, the diffusion coefficient

TABLE 7

The variation of k/r_o^2 ($min^{-1} \times 10^3$) with time after the indicated corrections of the sucrose loss for an initial rapid phase (R. catesbiana)

TEMP.	TIME	CORRECTION FACTOR (PER CENT)			
		0	20	25	30
°C.	min.				
	5	5.6	1.6	...	...
	15	2.5	1.0	0.70	0.41
	30	1.9	0.92	0.73	0.50
25	60	1.4	0.90	0.73	0.58
	120	1.1	0.78	0.68	0.59
	240	0.8	0.63	0.58	0.52
	Mean:			0.68	
	15	1.4	0.36	0.21	...
	30	1.1	0.37	0.21	0.13
4	60	0.73	0.31	0.20	0.12
	120	0.53	0.28	0.19	0.13
	240	0.44	0.27	0.20	0.14
	Mean:			0.20	

TABLE 8

The variation of k/r_o^2 ($min^{-1} \times 10^3$) with time after the indicated corrections of the chloride exchange for an initial rapid phase (R. catesbiana)

TEMP.	TIME	CORRECTION FACTOR (PER CENT)			
		0	5	10	15
°C.	min.				
	5	2.4	1.8	1.3	0.8
	15	1.9	1.6	1.2	0.9
	30	1.8	1.6	1.4	1.1
25	60	1.8	1.7	1.5	1.3
	120	1.7	1.6	1.5	1.5
	240	1.4	1.4	1.4	1.3
	Mean:		1.6	1.4	
	15	1.3	1.01	0.77	0.53
	30	1.3	1.05	0.87	0.66
4	60	1.2	1.00	0.87	0.73
	120	0.9	1.04	0.96	0.88
	240	0.8	0.96	0.92	0.85
	Mean:		1.01	0.88	

for sucrose ($r_o = 0.05$ cm) is found to be 1.7×10^{-6} cm^2 min.$^{-1}$ at 25°C. and 0.5×10^{-6} cm^2 min.$^{-1}$ at 4°C. The corresponding values for chloride exchange are 3.5×10^{-6} and 2.2×10^{-6} cm^2 min.$^{-1}$. The emission of Cl^{36} into the sucrose Ringer's (fig. 5 B), gives constancy of k/r_o^2 for a correction of 5%, from which the diffusion coefficient of NaCl is 2.3×10^{-6} cm^2 min.$^{-1}$.

Comparison may now be made between these figures and data available for the free diffusion of ions in water. The chloride exchange coefficient is directly comparable with the diffusion coefficient of KCl. This follows from the well-known relation between the ion mobilities and the diffusibility of a salt: The diffusion coefficient of a strong electrolyte is proportional to twice the product of the cation and anion mobilities divided by their sum. Thus, the near identity of chloride and potassium mobilities makes KCl diffusibility a close measure of chloride mobility or exchangeability in aqueous solution.[13] In table 9 the data (k) for the bullfrog sciatic are compared with those obtained or calculated from available data (D) for free diffusion under comparable conditions of temperature and concentration. The rates in the nerve trunk are found to be about 1/300 that in free solution.

In the case of chloride, the effect of temperature is about the same as on free diffusion. This suggests that passage of this ion involves only the aqueous phase of the extracellular space rather than the permeability of structural elements. A similar conclusion may be drawn from the close agreement between k/D for sucrose at 4°C. and for NaCl at 25°C. with that for KCl at 25 and 4°C. At present no explanation can be offered for what appears to be a significantly larger k/D value

[13] That such is the case within precision limits of several per cent is shown by the following sample calculation: The "self-diffusion" (ionic exchange) of radioactive sodium gives a coefficient of 1.264 at 25°C. and in 1M NaI (Wang and Kennedy, '50). The diffusion coefficient of KCl at the same concentration level and temperature is 1.876 (Stokes, '50). Using these figures as the relative mobilities of sodium and chloride, we calculate the coefficient of NaCl as $2 \times \frac{1.264 \times 1.876}{1.264 + 1.876}$, or 1.51, as compared with the measured value of 1.483 (Stokes, '50).

for sucrose at 25°C. which is also reflected in the larger temperature coefficient for k.

The application of the same procedure for the evaluation of k for R. pipiens is open to greater question because of the complex geometry. And, indeed, tables set up as in tables 7 and 8, are not as decisive in delimiting a correction factor through constancy of k/r_o^2, although some improvement is to

TABLE 9

Diffusion coefficients (k), estimated for the fiber-containing core of R. catesbiana sciatics, and corresponding data for free aqueous diffusion (D) under similar conditions of concentration and temperature

SUBSTANCE	TEMP.	k	D	k/D	k_{25}/k_4	D_{25}/D_4
	°C.	$cm^2\ min^{-1} \times 10^6$	$cm^2\ min^{-1} \times 10^4$			
KCl	25	3.5	11.1 [1]	3.2×10^{-3}	1.6	1.75
	4	2.2	6.3 [2]	3.5×10^{-3}		
NaCl [3]	25	2.3	7.4 [3,4]	3.1×10^{-3}		
Sucrose	25	1.7	3.1 [5]	5.5×10^{-3}	3.4	1.71
	4	0.5	1.8 [2]	2.7×10^{-3}		

[1] Stokes ('50).

[2] From the 25°C. figure by application of a temperature correction derived from data in the International Critical Tables.

[3] Diffusion into 7% sucrose.

[4] Corrected for the relative viscosity (1.2) of a 7% sucrose solution (Gosting, personal communication).

[5] Gosting and Morris ('49).

be seen for about the same degree of correction as in R. catesbiana. If we take the mean k/r_o^2, as above, an appropriate r_o^2 must be employed to give k. For this purpose measurements were made at 1 to 2 mm intervals of the diameters of the 4 major branches as well as the trunk of 12 sciatic nerves, and a mean radius or mean square radius was evaluated using the relative lengths in order to weight the contribution of each segment. On this basis r_o^2 is 0.12 or 0.13×10^{-2}, depending on whether the mean is that of the radii or their squares. Using the larger value, the following coefficients are obtained: For

chloride exchange (KCl), 4.4×10^{-6}; for sodium-potassium exchange,[14] 2.9×10^{-6}; for sucrose diffusion, 1.2×10^{-6} cm^2 min.$^{-1}$. These values are therefore of the same order of magnitude as for the bullfrog sciatic nerve.

The above calculations show that passage into or out of the fiber-containing core could result from low coefficients in the interstitial space. From this standpoint the epineurium might not be the major source of diffusion resistance. This appears to be contrary to the considerable evidence now available that removal of the sheath greatly accelerates the rate that experimental effects are induced (see Crescitelli, '51, for references) and that ions penetrate or leave (Feng et al., '50; Shanes, '51b). However, the finding that desheathed trunks gain considerable weight, which analytical data indicate is due primarily to an increase in extrafibrillar volume (Shanes, '51b, and in press; see also Mullins and Grenell, '52),[15] provides an alternative explanation for the results of desheathing.

Thus, the early literature established that many cells are practically impervious to sugars and strong electrolytes. The osmotic effects described in table 4 demonstrate that such is the case in myelinated nerve. It follows that these agents must diffuse chiefly *between* the nerve fibers, hence the effective area available for entry into or exit from the intact trunk will be reduced in proportion to the sum of the fiber diameters divided by the circumference at any distance from the axis of the core; interfibrillar connective tissue will also reduce the available area. Consequently the increased rates of penetration resulting from sheath removal could be interpreted as the consequence of greater interfibrillar spacing and hence of effective diffusion area. The extent to which return of the sheath restored the original fiber spacing might determine the reversibility of the effect of desheathing.

[14] Corrected to a single nerve with the ratio of the coefficient for chloride exchange from single nerves to that for three nerves (= 1.32).

[15] Lorente de Nó (personal communication, and '52) has confirmed histologically the increase in extrafibrillar space.

However, the recent finding (Shanes, in preparation) that the loss of radioactivity from the lumen of an otherwise empty bullfrog sheath duplicates that from intact nerve eliminates any doubt that the epineurium is the major barrier. The diffusion coefficient of the sheath, previously estimated from the initial component, can be shown to account for the slow component of the emission curve. The values for k given above may therefore be regarded as virtual rather than real, providing as they do the *apparent* diffusion or exchange coefficients of the core. The much larger CO_2 diffusion coefficient (Fenn, '28) is probably indicative of the greater penetrability of "non-polar" substances.

It is clear that diffusion and ionic exchange in the extracellular spaces of frog nerve are more complex than hitherto appreciated. But there can be little doubt that strong electrolytes and "polar" substances, generally, enter and leave the nerve trunk slowly under usual experimental conditions. This must be taken into account in evaluating earlier studies and in future attempts to investigate the more active aspects of ionic transfer.

It is a pleasure to acknowledge our indebtedness to the many who have so generously made available additional equipment which expedited this study: To Drs. Howard L. Andrews and Charles Maxwell, of the Laboratory of Physical Biology, for a complete Tracerlab Automatic Counting Unit; to Drs. Arthur T. Ness and Robert R. Williams, of the Laboratory of Pathology and Pharmacology, for a Nuclear Instrument and Chemical Corp. scaler and for the preamplifier from a Nuclear Measurements Proportional Counter Converter. We are also indebted to Mr. Charles J. Byrne and his staff of the Laboratory Aids Branch for their wholehearted cooperation in meeting our instrumental problems.

SUMMARY

The subject of this study has been ionic exchange — potassium for sodium, Cl^{36} for the inactive ion — and the diffusion of NaCl and of low concentrations of C^{14} labeled sucrose into and out of the sciatic nerves of R. pipiens and R. catesbiana. The nerve "space" occupied by sucrose is smaller than for chloride; this is in keeping with the greater osmotic effectiveness of excess sucrose than NaCl. The potassium space equals the chloride space, which is consistent with the identity of the osmotic effects of KCl and NaCl. Complete replacement of sodium in the medium by potassium causes a large gain in weight due to uptake of the medium, which does not occur when sucrose or choline are used as substitutes. Replacement of only 1/3 of the sodium with potassium leaves the water balance undisturbed.

The exit of the experimental agents from nerve occurs in two stages: An initial, smaller, rapid one succeeded by a larger prolonged one. The first component is bigger for sucrose than for chloride, while the second is slower for the sugar. Reduction of the temperature from 25 to 4°C. slows the emission of sucrose relatively more than that of chloride.

These observations suggest that the extracellular space is at least a two phase system. The fast component appears accounted for by the sheath (epineurium), the slow by the fiber-containing core. On this basis the diffusion and exchange coefficients of the core are estimated to be about 1/300 of the values for free aqueous diffusion.

LITERATURE CITED

BOYLE, P. J., AND E. J. CONWAY 1941 Potassium accumulation in muscle and associated changes. J. Physiol., *100:* 1.

CRESCITELLI, F. 1951 Nerve sheath as a barrier to the action of certain substances. Am. J. Physiol., *166:* 229.

FENG, T. P., C. H. HSU AND Y. M. LIU 1950 Correlation of potassium movement into and out of the nerve with its depolarization and repolarization. Chin. J. Physiol., *17:* 281.

FENN, W. O. 1928 The carbon dioxide dissociation curve of nerve and muscle. Am. J. Physiol., *85:* 207.

FENN, W. O., D. M. COBB, A. H. HEGNAUER AND B. S. MARSH 1934 Electrolytes in nerve. Am. J. Physiol., *110:* 74.

GOSTING, L. J., AND MARGARET S. MORRIS 1949 Diffusion studies on dilute aqueous sucrose solutions at 1 and 25° with the Gouy interference method. J. Am. Chem. Soc., *71:* 1998.

HILL, A. V. 1928 The diffusion of oxygen and lactic acid through tissues. Proc. Roy. Soc. Lon. B, *104:* 39.

LORENTE DE NÓ, R. 1950 The ineffectiveness of the connective tissue sheath of nerve as a diffusion barrier. J. Cell. and Comp. Physiol., *35:* 195.

———— 1952 Potassium and sodium ions in nerve physiology. Cold Spr. Harb. Symp. Quant. Biol., *17:* 299.

MULLINS, L. J., AND R. G. GRENELL 1952 The influence of the axon sheath on phosphate diffusion. J. Cell. and Comp. Physiol., *39:* 164.

RENYI, G. ST. DE 1929 The structure of cells in tissues as revealed by microdissection. II. The physical properties of the living axis cylinder in the myelinated nerve fiber of the frog. J. Comp. Neur., *47:* 405.

ROBINSON, C. V. 1950 Windowless, flow type, proportional counter for counting C^{14}. Science, *112:* 198.

SHANES, A. M. 1946 A neglected factor in studies of potassium distribution in relation to the resting potential of nerve. J. Cell. and Comp. Physiol., *27:* 115.

———— 1948a Metabolic changes of the resting potential in relation to the action of carbon dioxide. Am. J. Physiol., *153:* 93.

———— 1948b Medium concentration in relation to the water content and electrical properties of nerve. Fed. Proc., *7:* 111.

———— 1950a Potassium retention in crab nerve. J. Gen. Physiol., *33:* 643.

———— 1950b Drug and ion effects in frog muscle. J. Gen. Physiol., *33:* 729.

———— 1951a Potassium movement in relation to nerve activity. J. Gen. Physiol., *34:* 795.

———— 1951b Factors in nerve functioning (Symposium). Fed. Proc., *10:* 611.

———— 1952 Ionic transfer in nerve in relation to bio-electrical phenomena. New York Acad. Sci., *55:* 1.

———— 1953 Effects of sheath removal on bullfrog nerve. J. Cell. and Comp. Physiol., *41:* 305.

STOKES, R. H. 1950 The diffusion coefficients of 8 uni-univalent electrolytes in aqueous solution at 25°. J. Am. Chem. Soc., *72:* 2243.

WANG, J. H., AND J. W. KENNEDY 1950 Self diffusion coefficients of sodium ion and iodide ion in aqueous sodium iodide solutions. J. Am. Chem. Soc., *72:* 2080.

EFFECTS OF OXYGEN CONCENTRATION ON X-RAY-INDUCED MITOTIC INHIBITION IN LIVING CHORTOPHAGA NEUROBLASTS [1]

MARY ESTHER GAULDEN, MARJORIE NIX AND JACK MOSHMAN
Biology Division and Mathematics Panel, Oak Ridge National Laboratory, Oak Ridge, Tenn.

SEVEN FIGURES

Irradiation of tissues under anaerobic conditions was shown by Crabtree and Cramer ('33) and Mottram ('35a, b) to result in a reduction of the inhibition of growth caused by radiation in mouse carcinoma and *Vicia* roots. These findings were confirmed by Read and Wolfe ('48) with *Vicia*, and by Hayden and Smith ('49) with barley, the work of the former authors indicating that the extent of inhibition was positively correlated with the amount of oxygen present at irradiation. Studying a specific aspect of growth, Anderson ('39) demonstrated that *Arbacia* eggs irradiated in hydrogen showed less delay in cleavage than those irradiated in air. Cohen ('39) found that radiation-induced delay in cleavage of *Arbacia* eggs was reduced when the eggs were irradiated while closely packed in capillary tubes, a condition which most probably led to reduced oxygen tension. These findings clearly show that the effects of radiation on growth are influenced by oxygen.

The present investigation was initiated to determine the effects of oxygen concentration on radiation-induced mitotic inhibition in grasshopper neuroblasts (Gaulden and Nix, '50). These cells can be grown *in vitro*, and the mitotic rate of large numbers of them can be obtained with relative ease over a period of time after irradiation. Moreover, the abnormalities

[1] Work performed under Contract No. W-7405-Eng-26 for the Atomic Energy Commission.

in cell structure, which appear to be partly or wholly responsible for the abnormal mitotic activity of neuroblasts following exposure to x rays, have been determined (Carlson, '42; Wolfson, '51; Gaulden, unpublished). Consequently, the effects of oxygen on mitotic response to x rays could be studied in detail and interpreted in light of alterations, if any, in the abnormalities of cell structure and behavior caused by radiation. It was hoped that such a study might help bring the mechanism of the oxygen effect into sharper focus.

The mitotic response of neuroblasts to x rays was found to be influenced by the concentration of oxygen present during irradiation, the period of recovery being more sensitive to the effect of oxygen than the period of initial depression of mitosis.

MATERIALS AND METHODS

Three doses of x rays were used. Doses of 3.5 and 8 r were chosen because they do not depress mitotic activity to zero; therefore, measurement can be made of differences in depth of mitotic depression after irradiation at different concentrations of oxygen. A dose of 64 r, on the other hand, depresses mitosis to zero, resulting in successive periods of minimum and maximum activity (recovery) that are of considerable duration and therefore are readily measurable. A General Electric Maxitron 250 kvp unit was used for the experiments with 3.5 and 8 r: at 100 kvp, with 3.5 mm of aluminum filtration, the intensities were, respectively, 6 r/minute at 90-cm target distance and 15 ma and 8 r/minute at 96-cm target distance and 25 ma. For those experiments involving a dose of 64 r, a General Electric Maximar unit was used. The factors were 125 kvp, 15 ma, and 70-cm target distance. A 0.5-mm aluminum filter was added to the inherent filtration of the machine (equivalent to 3 mm of aluminum). The intensity was 32 r/minute. Intensities were measured in air with 25-r and 100-r Victoreen thimble ionization chambers.

Irradiation was carried out at 6 different concentrations of oxygen: zero (nitrogen, carbon dioxide, or vacuum), 2, 5,

10, 21 (air), and 100% oxygen. Helium served as the inert gas in the mixtures, the accuracy of percentage of oxygen being ± 0.2 (analysis by vendor: Ohio Chemical and Surgical Equipment Co.) except in the case of one cylinder of 10% oxygen. Since it would have been very difficult to irradiate the grasshopper eggs [2] at all 6 concentrations at the same time, two sets of eggs were simultaneously irradiated at two concentrations of oxygen. Eight pairs of oxygen concentrations were used: zero (carbon dioxide) and 21%; zero (nitrogen) and 21%; zero (vacuum) and 2%; 2 and 10%; zero (vacuum) and 5%; zero (vacuum) and 10%; 21 and 100%; and zero (vacuum) and 100% oxygen.

For irradiation at two given oxygen concentrations, moistened *Chortophaga* eggs (14 days old at 26°C.) were placed in two adjacent containers made of polystyrene (fig. 1 a). Lids were sealed on the containers with vacuum grease. One exit tube of each container was connected to a vacuum line and the other to a tank of gas containing the desired concentration of oxygen (fig. 1 b). The containers were alternately evacuated to a few millimeters of mercury and flushed with gas three times. This operation (completed within three minutes [3]) was necessary in order to be reasonably sure that the atmosphere surrounding the eggs during irradiation was the intended one. Manometers permitted a check on pressure. Within one minute x radiation followed flushing, and lasted for 35, 60, or 120 seconds, depending on the dose. Except when irradiated *in vacuo,* eggs were irradiated at a few millimeters positive pressure to allow detection of leaks. Eggs were subjected to 4 experimental conditions without irradiation: evacuation plus flushing with carbon dioxide, with air, or with 100% oxygen, and evacuation alone. All experiments with and without irradiation, were performed at room temperature (ca 25°C.).

[2] The term "egg" is herein used to denote the intact chorion and its contained yolk, embryo, and serosal and amnionic membranes.

[3] When eggs were irradiated in a vacuum the container was evacuated for three minutes before irradiation.

Immediately after treatment the containers were opened and the eggs placed in 70% alcohol for one-minute sterilization. The eggs were dried on sterile filter paper and transferred to artificial culture medium (Carlson, Hollaender and Gaulden, '47) where the embryos were separated from the yolk and embryonic membranes. Appendages were cut off to expose the ventral surface and the head and part of the abdomen cut away to reduce the tissue mass. Each dissected

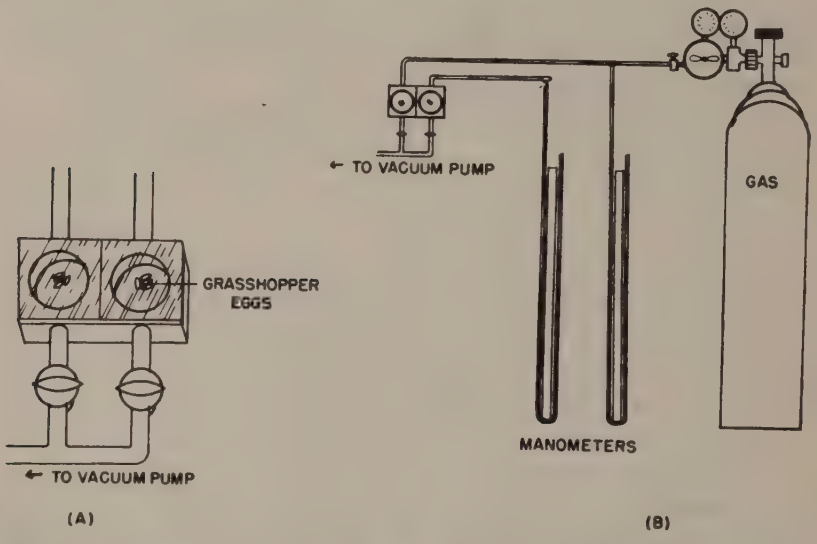


Fig. 1 A, Containers for exposing eggs. B, Set-up for irradiating one set of eggs in vacuum and one set in a given concentration of oxygen.

embryo was mounted, in a drop of medium, ventral side against a cover glass, which was then inverted and sealed over a depression slide with thick mineral oil. All operations were carried out under aseptic conditions (Carlson, Hollaender and Gaulden, '47).

The hanging-drop preparations were studied with a microscope, the lower portion of which was enclosed in an incubator maintained at a constant temperature of $38° \pm 1°C$. All embryos in an experiment were placed in the incubator 21 minutes after irradiation, and observations were begun

one minute later. Neuroblasts lie at the ventral surface of the grasshopper embryo and, therefore, are readily accessible in the hanging-drop preparations for observation with the oil immersion lens of the microscope. Heat was filtered from the microscope illumination by means of a copper sulfate solution.

The data were obtained in the following way. The duration of mid-mitosis — prometaphase, metaphase, and anaphase — in the neuroblast at 38°C. is 22 minutes, a period which is almost constant and which is unaffected by the doses of radiation used. Consequently, by recording the number of cells in mid-mitosis at 22-minute intervals after irradiation, a quantitative determination of the number of cells going through mitosis in a given period of time can be obtained. Approximately 250 cells per embryo (all the neuroblasts in 6 of the body segments) were examined at 17 22-minute intervals, i.e., over a period of 374 minutes [4] following irradiation, and the numbers of cells in mid-mitosis recorded. For each experiment comparing two concentrations of oxygen, 12 embryos or 3000 cells irradiated at each concentration were studied. Exceptions were the zero (vacuum)-2% oxygen experiment with 8 embryos and the zero (nitrogen)-21% oxygen experiment with 23 embryos irradiated at each concentration to give totals of about 4000 and 11,500 neuroblasts, respectively, examined in the two experiments. In those experiments without irradiation, 12 untreated embryos and 12 treated under each of the previously mentioned conditions were studied. Because of the short interval between observations, data from only 4 to 6 embryos could be recorded at one time. The data of each comparison of oxygen concentrations were compiled, therefore, from the data obtained from 4 to 8 sets of embryos irradiated on 4 to 8 successive days.

It should be emphasized that the concentrations of oxygen designated for the experiments are those which surrounded the tough chitinous chorion enclosing the embryo *(in vivo)*.

[4] This represents the approximate period of optimum growth of embryos in hanging-drop preparations.

The results of Gaulden, Carlson and Tipton ('49) make it seem highly unlikely that these concentrations (excepting air) were reached at the level of the cells inside the chorion. These authors separated embryos from the embryonic membranes and yolk and exposed them *in vitro*, to different gases. Within 30–60 seconds after pure carbon dioxide or nitrogen was introduced into hanging-drop preparations, the spindles of neuroblasts in metaphase were either partially or completely destroyed. Further, the chromatin of all stages became highly refractive within a few minutes, and in the case of middle and late prophase nuclei, it reverted to a state resembling that of early mitotic nuclei. In other words, neuroblasts in direct contact with carbon dioxide or nitrogen were extremely sensitive to these gases in that they suffered immediate and rather drastic cytological alterations. We have found that the neuroblasts of embryos exposed *in vivo* to the same gases for periods of time up to two hours exhibit no detectable cytological aberrations. It therefore seems reasonable to assume, in the present experiments in which embryos were treated *in vivo* and for only a few minutes, that the oxygen concentration at the cellular level during irradiation did not reach the concentration prevailing outside the chorion. Needless to say, the extreme sensitivity of neuroblasts exposed directly to anaerobic conditions precludes studying their response to x rays under these conditions.

STATISTICAL ANALYSES AND RESULTS

Analysis of the data was approached in two ways. First, each experiment was analyzed separately so that direct comparison could be made of the reaction of two groups of cells to irradiation given under exactly the same conditions except for concentration of oxygen surrounding the cells. This analysis, made after completion of each experiment, served as a guide for further experimentation. Second, the data of all experiments with a dose of 64 r were combined and, together with the 3.5- and 8-r experiments, were used to evaluate the

effects of oxygen on certain phases of mitotic response to radiation.

Analysis of individual experiments

The results of some of the experiments in which two groups of eggs were simultaneously irradiated at two tensions of oxygen are presented graphically in figures 2–5.[5] The standard error of each point is indicated. It can be seen that, irrespective of oxygen tension, the mitotic activity of the neuroblasts follows the same general pattern characteristic of mitotic cells given moderate doses of irradiation: the number of mid-mitotic cells decreases to a minimum and then progressively increases to overshoot and gradually to return to the normal level of activity. There is some indication, however, that the extent of the mitotic response differs with oxygen concentration, especially in the 64-r experiments. The data have, therefore, been subjected to statistical analysis to determine the significance of the differences.

A biometrical analysis of each of the 11 paired experiments — one each with 3.5 and 8 r and 9 with 64 r of x rays — was made to determine whether the differences between effects of two given oxygen concentrations on mitotic response to radiation were significant. The raw data were transformed according to the procedure of Freeman and Tukey ('50) and then analyzed by means of the analysis of variance test (Fisher, '46). (The data have been omitted to conserve space.) In table 1 are shown the probability levels of the F ratios thus obtained for determining the statistical significance of (1) main effects, i.e., the differences in the total number of cells completing mitosis within 374 minutes after irradiation at two concentrations of oxygen, and (2) interaction, i.e., the differences after adjustment for over-all effects, between the number of mitotic cells at each of the 17 22-minute intervals in embryos irradiated at one concentration as compared with the number at comparable intervals in embryos irradiated at

[5] The average number of mid-mitotic cells per embryo in the absence of x rays is about 10.

the other concentration of oxygen. The reaction of the neuroblasts to 64 r of x rays differed with oxygen concentration in a statistically significant manner with respect to both criteria in the following experiments: nitrogen-21%, carbon dioxide-21%, 2–21%, vacuum-5% (fig. 2), vacuum-10%, and vacuum-100% (fig. 3). In the 21–100% experiment with 64 r of x rays, as well as in the vacuum-100% experiment with 8 r (fig. 4), there was a statistically significant difference between the total number of cells completing mitosis after irradiation

TABLE 1

Probability levels of F ratios obtained by analysis of variance test of individual experiments. The dose of radiation was 64 r except as indicated

SOURCE OF VARIATION \ OXYGEN CONC. (%)	N_2-21	CO_2-21	2-21	Vac-5	Vac-10	Vac-100	21-100	Vac-100 (8 r)	Vac-100 (3.5 r)	Vac-2	2-10
Total number of mitotic cells	++	++	++	++	++	++	+	+	—	—	—
Interaction	++	++	++	++	++	++	—	—	—	—	—

++ Significant at 0.01 probability level.
+ Significant at 0.05 probability level.
— Not significant at 0.05 probability level.

at the different concentrations of oxygen; the differences observed between concentrations at individual observation intervals were not, however, significant at the 0.05 level of probability in these two experiments. There was no statistically significant difference in the way cells reacted to 3.5 r of x rays in vacuum, as compared with 100% (fig. 5) or to 64 r of x rays in vacuum as compared with 2% oxygen or in 2% as compared with 10% oxygen.

In view of the statistically significant difference obtained between vacuum and 5% oxygen (64 r) (fig. 2), the results obtained between 2 and 10% oxygen (64 r) — no statistically significant difference — seem out of line. The cylinder of 10%

oxygen used in this experiment was inadvertently returned to the vendor before its contents could be analyzed. Results of the experiment suggest that it did not contain the specified concentration of oxygen.

Biometrical analysis of the data obtained from neuroblasts subjected to experimental conditions without irradiation was made in the same way as that described for the radiation experiments. It revealed that the mitotic rate of neuroblasts subjected to evacuation plus flushing with carbon dioxide, with air, or with oxygen or evacuation alone did not differ in a statistically significant manner from the mitotic rate of neuroblasts which had received no treatment whatsoever. We can conclude that, under the conditions used, neither the lowering and raising of gas pressure nor the concentration of oxygen about the eggs have any detectable effect on neuroblast mitosis in the absence of radiation.

Analyses for effects of oxygen on certain phases of mitotic response to radiation

There are 4 phases of the mitotic response of neuroblasts to x radiation which are amenable to quantitative measurement: (1) the depth to which mitosis is depressed, (2) the duration of the period of minimum activity, (3) the time at which maximum mitotic activity occurs during recovery and (4) the total number of cells which go through mitosis within a given period of time following irradiation.

Depth of mitotic depression. Figures 4 and 5 show that irradiation with 3.5 and 8 r of x rays in 100% oxygen results in a slightly greater depression of mitosis (66 minutes after irradiation) than irradiation in vacuum. This difference was evaluated by Student's "t" test and was found *not* to be statistically significant. In view of the possible non-normality of the data, several non-parametric, two-sample tests were employed as a further check, all with negative results — the extent to which mitosis is initially depressed by x radiation is affected only slightly, or not all all, by these two widely separated oxygen tensions.

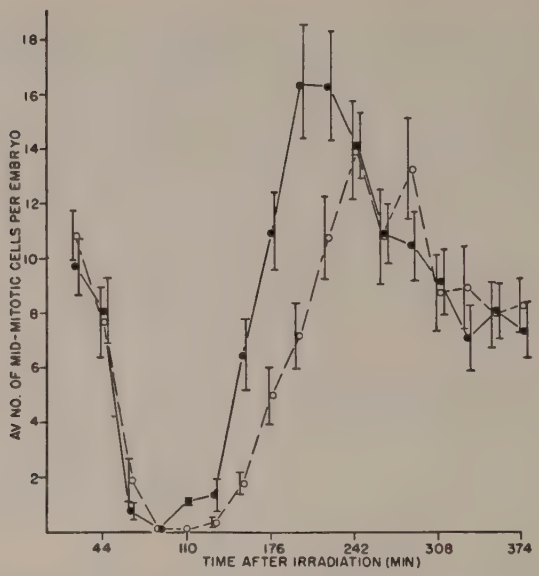


Fig. 2 Effects of 64 r of x rays on mitotic count of neuroblasts irradiated in vacuum and 5% oxygen. ——— ●, vacuum - - - - - ○, 5% oxygen.

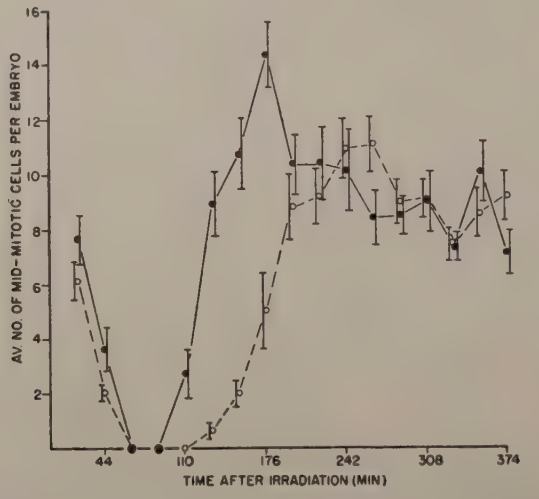


Fig. 3 Effects of 64 r of x rays on mitotic count of neuroblasts irradiated in vacuum and 100% oxygen. ——— ●, vacuum - - - - - ○, 100% oxygen.

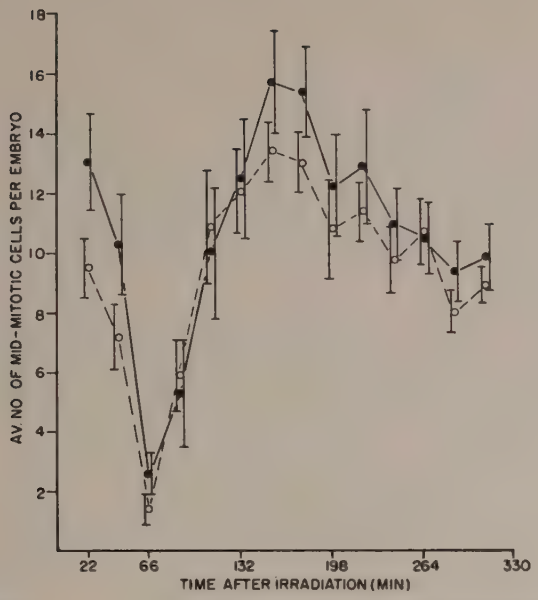


Fig. 4 Effects of 8 r of x rays on mitotic count of neuroblasts irradiated in vacuum and 100% oxygen. ——— ●, vacuum – – – – ○, 100% oxygen.

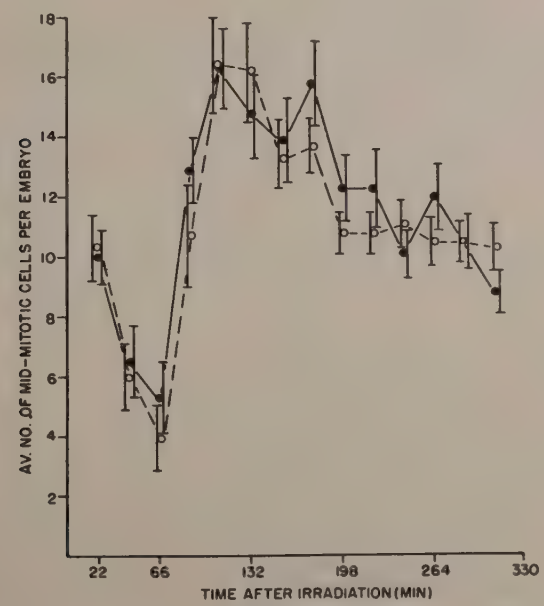


Fig. 5 Effects of 3.5 r of x rays on mitotic count of neuroblasts irradiated in vacuum and 100% oxygen. ——— ●, vacuum – – – – ○, 100% oxygen.

Duration of period of minimum mitotic activity. The mean duration of the period during which there are no cells in mid-mitosis after irradiation was computed from the raw data for all embryos given 64 r of x rays at each of the 6 concentrations of oxygen used. The region of 95% confidence limits about each mean was computed in the usual manner (Eisenhart et al., '47).

The results are presented graphically in figure 6 a. A weighted least-squares line was fitted to the first 4 points and a weighted average was determined for the last two points. Intersection of the two linear segments occurs at about 13.2 $\pm$ 2.2% [6] oxygen. Thus, we see that the duration of the radiation-induced period of minimum mitotic activity increases with increasing concentration of oxygen and levels off at about 13%.

Time of maximum mitotic activity. The untransformed mean intervals between irradiation and the time at which the peak of mitotic activity occurred in all embryos treated at each concentration of oxygen are presented in figure 6 b. The 95% confidence limits for each mean interval were computed as described above for duration of minimum activity period. A line has been fitted by weighted least squares to the mean intervals obtained for the three lower concentrations of oxygen. The weighted average of the intervals for the three higher concentrations was computed. These two line segments intersect at about 6.8 $\pm$ 0.4% [6] oxygen. Thus, the interval between irradiation and maximum activity increases with increasing oxygen concentration up to about 7%, above which there is no further increase.

Number of mitoses. The effect of different oxygen concentrations on the number of cells going through mitosis within 374 minutes following irradiation is expressed as a ratio of the total number of mitotic cells observed after irradiation at a given oxygen concentration to the total observed after irradiation at 0% oxygen. Ratios were calculated using only

[6] The figure following the $\pm$ symbols represents approximately a 95% confidence interval.

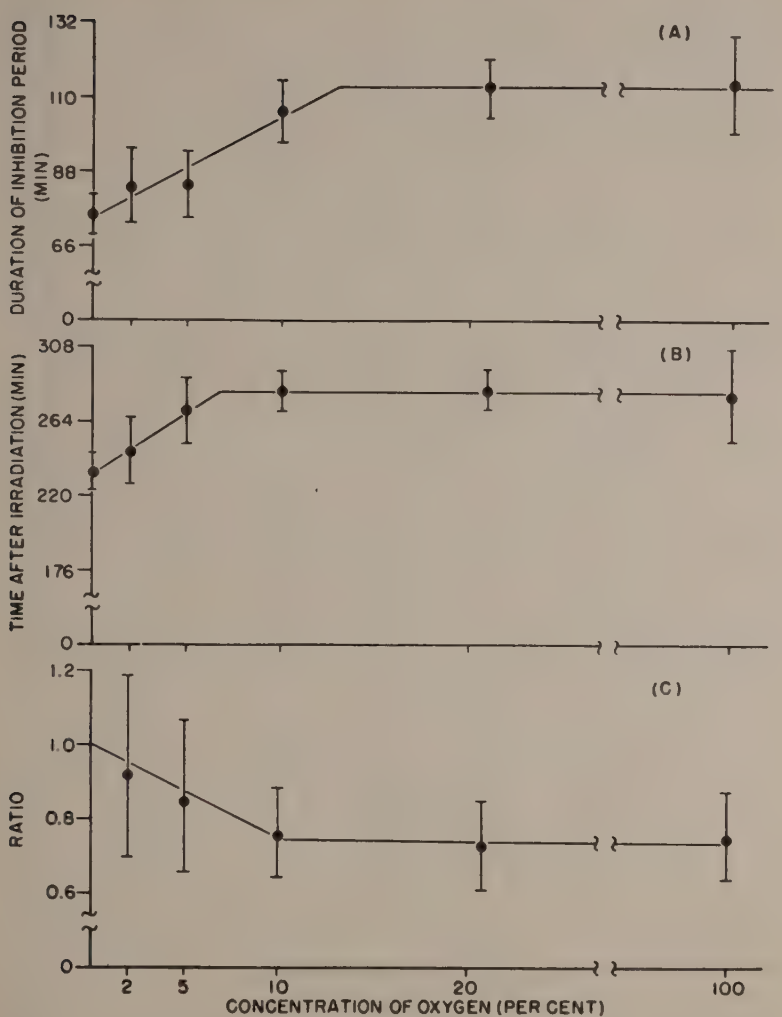


Fig. 6 a, Duration of inhibition period as a function of oxygen concentration. b, Time of maximum mitotic activity as a function of oxygen concentration. c, Total number of mitotic cells within 374 minutes after irradiation as a function of oxygen tension (expressed as the ratio of the number of cells at a given concentration of oxygen to the number at 0% oxygen [vacuum]).

The interval about each point represents the region within the 95% confidence limits.

those experiments in which embryos had been irradiated simultaneously at 0% oxygen and at the oxygen concentration specified. In computing the confidence limits for the ratios the method of Geary ('30) was used. Since a ratio is generally subject to more variation than the numerator and denominator individually, the confidence limits are relatively wide.

The ratios have been plotted against oxygen concentration in figure 6 c and line segments fitted by the weighted least squares to the points. The points at 10 and 21% oxygen represent the average of two experiments for each concentration. The number of cells completing mitosis within 374 minutes after irradiation decreases as the oxygen concentration increases up to $10.0 \pm 0.2\%$ [6] and then levels off.

It is of interest that the effects of radiation on mitosis increase rapidly with increasing oxygen concentration up to about 10% at which point they level off abruptly. Baker and Edington ('52), studying the frequencies of x-ray-induced translocations and recessive lethals in *Drosophila* at varying tensions of oxygen have found that the frequencies of genetic effects increase sharply as oxygen concentration rises to about 11%, but increasing tension above this level causes little or no further increment in the frequencies of effects produced by a given dose of x rays. These authors replotted the data of Giles and Beatty ('50) and Giles and Riley ('50) and found that, in *Tradescantia*, the frequency with which x-ray-induced chromosome interchanges occur bears a similar relationship to oxygen concentration. Just why the influence of oxygen concentration changes so abruptly at approximately the 10% level is not understood but is possibly related to concentration in the tissues.

DISCUSSION

The results of previous studies on the influence of oxygen on biological action of radiation have been interpreted in one of two ways: (1) oxygen alters the quantity of radiation damage (Thoday and Read, '49; Giles and Beatty, '50; Giles and

[6] The figure following the $\pm$ symbols represents approximately a 95% confidence interval.

Riley, '50; King, Schneiderman and Sax, '52); (2) oxygen modifies the quality as well as the quantity of radiation injury (Schwartz, '52). A review of the alterations in cell structures and in mitotic rate, which are responsible for the changes in mid-mitotic cell frequency after x radiation, is pertinent to an interpretation of the effects of oxygen in modifying the response of grasshopper neuroblasts to x rays.

Preliminary work by Wolfson ('51) and Gaulden (unpublished) indicates that, after a dose of 64 r of x rays, approximately half the neuroblasts treated in late prophase proceed to prometaphase with little delay. Of the remaining late prophases, some are retarded so that they do not reach mid-mitosis until 22–88 minutes after irradiation, while some undergo such drastic changes that they are delayed for considerably longer periods of time. The chromatin of these latter cells, as well as that of the majority of cells irradiated in middle prophase, gradually resumes the appearance of early prophase, very early prophase or interphase chromatin,[7] i.e., the nuclei appear to revert to the early stages of mitosis. Consequently, for a while there are no cells entering mid-mitosis. The reverted cells gradually recover and resume their progress through mitosis, along with cells irradiated in early mitosis, whose progress was retarded little or none. Following the period of minimum activity, therefore, the frequency of mid-mitotic cells increases and overshoots the normal level, to reach a maximum, after which it returns to normal. The reaction of neuroblasts to 8 r and less of x rays is of a similar but less drastic nature. (Cell structures and mitotic progress of prometaphase, metaphase, and anaphase cells are unimpaired by 64 r and less of x rays.)

Thus we see that, at the doses used in the present study, reversion of middle and late prophase nuclei to earlier stages of mitosis is the event primarily responsible for radiation-induced mitotic inhibition. The depth to which mitosis is depressed by irradiation is determined by the number of cells which undergo reversion. Although it is very difficult to sepa-

[7] See Carlson and Hollaender ('48) for description of mitotic stages.

rate injury and recovery in terms of cell activity, we feel that this response can be considered, in the main, to be a manifestation of the initial damage of x rays to the cell. The duration of the period of minimum activity is determined by how far back into early mitosis the cells revert and how long they remain in the reverted condition. It represents in part the extent of injury produced and in part the rate with which the cells begin to make demonstrable recovery. The time and magnitude of maximum activity depend on the proportion of reverted cells which recover and on the rate at which they

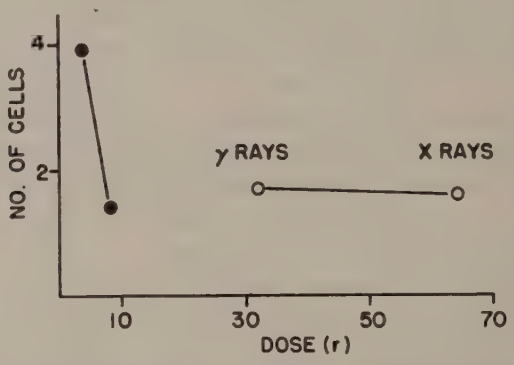


Fig. 7 Relationship of effects of radiation on mitosis to the dose rate.
———●, Number of cells at depth of depression.
– – – – ○, Number of cells (× 10) in the recovery period (110–198 minute period after irradiation).

recover (reach mid-mitosis). The total number of neuroblasts that go through mitosis during 374 minutes after irradiation gives some indication of the combined extents of injury and recovery.

If the sensitivity of mitotic response to an increase in dose of radiation, i.e., to an increase, presumably, in amount of injury, is examined, it will be seen that increasing the dose by a factor of about two results in a considerable increase in the depth to which mitosis is depressed (fig. 7). Increasing the dose of radiation by a factor of two has only a small effect, however, on the number of cells that recover following the

inhibition period (fig. 7). (The comparison of γ rays [Carlson, Snyder and Hollaender, unpublished] with x rays made in figure 7 seems valid in view of the fact that 64 r of γ rays alters mitotic activity to about the same extent as does 64 r of x rays.) Thus depression of mitotic activity is considerably more sensitive to change in dose of radiation than is the number of cells showing recovery.

The present work shows that the concentration of oxygen present during irradiation has little or no effect on the depth to which mitotic activity of neuroblasts is depressed by radiation. On the other hand, the duration of the period of minimum activity, the time of maximum activity, and the total number of cells going through mitosis reflect a more adverse effect of radiation when cells are irradiated at the higher than at the lower concentrations of oxygen. It would appear, therefore, that oxygen does not influence the number of neuroblasts affected by radiation but does influence the extent of recovery. In other words, alteration of oxygen concentration does not seem to act in the same way as a change in the effective dose of radiation.

The work of Schwartz ('52) on maize suggests that oxygen modifies the quality and not the quantity of initial radiation injury. He compared the frequencies of mutant kernels arising from breakage with and without reunion. If the effect of oxygen is on the amount of initial breakage, one would expect that the two classes of mutant kernels would occur in the same proportions at different concentrations of oxygen. Schwartz has demonstrated, however, that the ratio of the two classes of mutant kernels derived from pollen irradiated in air (21% oxygen) differs significantly from the ratio obtained in kernels derived from pollen irradiated in nitrogen. This finding has led him to postulate that "chromosomes broken in the absence of oxygen are more capable of rejoining than chromosomes broken in the presence of oxygen." McClintock ('41) has suggested that the conditions under which chromosomes are broken may affect their subsequent behavior. On the basis of results of experiments with bacteriophage,

Hewitt and Read ('50) suggest that point gene mutations are not influenced by oxygen tension, but that mutations arising from chromosome aberrations are subject to oxygen effect. Baker and Edington ('52) have noted that the effects of oxygen on x-ray-induced recessive lethals and translocations in *Drosophila* can be interpreted in a similar manner.

The effects of oxygen on radiation-induced chromosomal aberrations in maize, recessive lethals and translocations in *Drosophila,* and mitotic response of grasshopper neuroblasts indicate that the influence of oxygen on radiation effects involves more than merely a change in the amount of injury produced. The exact nature of the oxygen effect defies explanation at the present time, because not even the ultimate factors involved in the biological action of the radiation itself are well understood.

Although the criterion for determining the extent of initial radiation damage in the neuroblast — depth of mitotic depression — seems a reliable one, especially in view of its sensitivity for detecting differences in effects of doses of radiation differing by only a few r, the possibility is not to be ignored that oxygen may alter the amount of damage produced by radiation. The effects of oxygen tension on radiation-induced reversion of prophase cells, which results in mitotic depression, are being studied in considerable detail in the neuroblast in order to obtain more exact quantitative and qualitative data on this response to radiation. It is hoped that through such a study the action of oxygen with respect to injury and recovery can be further elucidated.

SUMMARY

1. Embryos of the grasshopper, *Chortophaga viridifasciata,* were exposed, *in vivo,* to 3.5, 8, or 64 r of x rays at different concentrations of oxygen: zero (nitrogen, carbon dioxide, or vacuum), 2, 5, 10, 21 (air), and 100% oxygen. Following irradiation the embryos were made into hanging-drop preparations and mid-mitotic counts of the living neuroblasts were made at 22-minute intervals over a period of 6 hours.

2. Neuroblast mitosis *in vivo* is not affected by oxygen tension in the absence of radiation under the experimental conditions used.

3. The influence of oxygen tension on 4 phases of mitotic response of neuroblasts to x rays was determined. The depth to which mitosis is depressed by x rays is affected only slightly or not at all by the oxygen present during irradiation. At concentrations of oxygen between 0 and 10%, the duration of the period of minimum mitotic activity and the interval of time between x raying and maximum mitotic activity are both positively correlated with oxygen concentration. The number of cells going through mitosis within 6 hours of treatment is negatively correlated with oxygen concentration. The effects of oxygen concentrations of 21 and 100% are not significantly different from those obtained with 10% oxygen.

4. The fact that neuroblasts do not respond to change in oxygen tension (at irradiation) in the same way as to a change in dose of radiation suggests that the influence of oxygen tension on the response of these cells to radiation involves more than merely a change in the amount of injury produced by the radiation.

ACKNOWLEDGMENTS

The counsel of Dr. J. Gordon Carlson and the technical assistance of Mr. George J. Atta are gratefully acknowledged.

LITERATURE CITED

ANDERSON, R. S. 1939 The X-ray effect on the cleavage time of *Arbacia* eggs in the absence of oxygen. Biol. Bull., *77:* 325.

BAKER, W. K., AND C. W. EDINGTON 1952 The induction of translocations and recessive lethals in *Drosophila* under various oxygen concentrations. Genetics, *37:* 665–667.

CARLSON, J. G. 1942 Immediate effects of 31 r of X-rays on the different stages of mitosis in neuroblasts of *Chortophaga*. J. Morph., *71:* 449–462.

CARLSON, J. G., AND A. HOLLAENDER 1948 Mitotic effects of ultraviolet radiation of the 2250 Å region, with special reference to the spindle and cleavage. J. Cell. and Comp. Physiol., *31:* 149–173.

CARLSON, J. G., A. HOLLAENDER AND M. E. GAULDEN 1947 Ultraviolet radiation as a means of sterilizing tissue culture materials. Science, *105:* 187–188.

COHEN, IRVING 1939 Cleavage delay in *Arbacia punctulata* eggs irradiated while closely packed in capillary tubes. Biol. Bull., *77:* 324–325.

EISENHART, C., M. W. HASTAY AND W. A. WALLIS (editors) 1947 Techniques of Statistical Analysis. McGraw-Hill Book Co., New York, N. Y., pp. 397–408.

CRABTREE, H. G., AND W. CRAMER 1933 The action of radium on cancer cells. II. Some factors determining the susceptibility of cancer cells to radium. Proc. Roy. Soc. (London), B *113:* 238–250.

FISHER, R. A. 1946 Statistical Methods for Research Workers, 10th ed. Oliver and Boyd, London.

FREEMAN, M. F., AND J. W. TUKEY 1950 Transformations related to the angular and the square root. Ann. Math. Stat., *21:* 607–611.

GAULDEN, M. E., J. G. CARLSON AND S. R. TIPTON 1949 Effects of carbon dioxide and nitrogen gas on mitosis as observed in living neuroblasts of grasshopper embryo. Anat. Rec., *105:* 16–17.

GAULDEN, M. E., AND M. NIX 1950 Effect of oxygen tension on x-ray induced mitotic inhibition. Genetics, *35:* 665–666.

GEARY, R. C. 1930 The frequency distribution of the quotient of two normal variates. J. Royal Stat. Soc., *93:* 442–446.

GILES, N. H., AND A. V. BEATTY 1950 The effect of X-irradiation in oxygen and in hydrogen at normal and positive pressures on chromosome aberration frequency in *Tradescantia* microspores. Science, *112:* 643–645.

GILES, N. H., AND H. P. RILEY 1950 Studies on the mechanism of the oxygen effect on the radiosensitivity of *Tradescantia* chromosomes. Proc. Nat. Acad. Sci., U. S., *36:* 337–344.

HAYDEN, B., AND L. SMITH 1949 The relation of atmosphere to biological effects of X rays. Genetics, *34:* 26–43.

HEWITT, H. B., AND J. READ 1950 Search for an effect of oxygen on the direct X ray inactivation of bacteriophage. Brit. J. Radiology, *23:* 416–423.

KING, E. D., H. A. SCHNEIDERMAN AND K. SAX 1952 The effects of carbon monoxide and oxygen on the frequency of x-ray induced chromosome aberrations in *Tradescantia.* Proc. Nat. Acad. Sci., U. S., *38:* 34–43.

MCCLINTOCK, B. 1941 The stability of broken ends of chromosomes in *Zea mays.* Genetics, *26:* 234–282.

MOTTRAM, J. C. 1935a On the alteration in the sensitivity of cells towards radiation produced by cold and by anaerobiosis. Brit. J. Radiology, *8:* 32–39.

———— 1935b Variations in the sensitivity of the cell to radiation in relation to mitosis. Brit. J. Radiology, *8:* 643–651.

READ, J., AND B. WOLFF 1948 The effect of the oxygen concentration on the sensitivity of the broad bean root to ionizing radiations. Brit. Emp. Cancer Report, *26:* 91–97.

SCHWARTZ, DREW 1952 The effect of oxygen concentration on X-ray-induced chromosome breakage in maize. Proc. Nat. Acad. Sci., *38:* 490–494.

THODAY, J. M., AND J. READ 1949 Effect of oxygen on the frequency of chromosome aberrations produced by alpha-rays. Nature, *163:* 133–134.

WOLFSON, NANCY D. 1951 Some studies on x-ray-induced blockage of mitosis in grasshopper neuroblasts. Thesis, University of Tennessee.

CHANGES IN BLOOD SUGAR AND TISSUE GLYCOGEN IN THE HAMSTER DURING AROUSAL FROM HIBERNATION [1]

CHARLES P. LYMAN AND ELIZABETH H. LEDUC

Department of Anatomy, Harvard Medical School, Boston, and Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts

SIX FIGURES

INTRODUCTION

The blood sugar and glycogen content of hibernating mammals has been the object of much study and has been ably reviewed by Suomalainen ('35) and Kayser ('50). All reports agree that the blood sugar of mammals in the hibernating state is lower than that of animals in the awake condition, although there has been considerable quantitative variation among the various investigations. It is also generally agreed that glycogen is used as a source of energy during the vigorous process of waking from hibernation, so that liver and muscle glycogen is lowered as the animal arouses (Weinland and Riehl, '08; Ferdmann and Feinschmidt, '32). On the other hand, there is little agreement concerning the amount of glycogen in the hibernating animal compared to that found in the non-hibernating state, nor have there been accurate quantitative measurements in the change of blood sugar and glycogen levels as an individual animal awakes from hibernation.

Preliminary investigations with golden hamsters in this laboratory indicated that the blood sugar content ran counter to the general rule and was not lower during the hibernating state than when the animal was awake. Furthermore, modern

[1] This research was supported in part by Air Force Contract No. A F 33(038)—18133 and in part by the Milton Fund of Harvard University.

microchemical and histochemical methods provide improved means of determining the blood sugar and tissue glycogen levels in animals in the hibernating state and of tracing the changes which take place in individual animals during the process of awakening. It therefore appeared profitable to re-examine glucose and glycogen levels during the hibernating and awake conditions, with particular emphasis on the waking period. In addition, the location of stored glycogen and the areas of its depletion have been reinvestigated by a recently developed histochemical method.

MATERIAL AND METHODS

Hamsters *(Mesocricetus auratus)* over three months old and of both sexes were used in the experiments. Control animals were taken from stock in an animal room maintained at $23° \pm 5°C$. Hibernation was induced by placing the animals in individual cages in a cold room maintained at $5° \pm 2°C$. as described in a previous paper (Lyman, '48). Control animals in the cold were similarly treated, but did not hibernate during the experiments.

Samples for liver and muscle glycogen determinations in the deeply hibernating hamster were made in the cold room by securing the animal to a board, making an incision in the abdomen and removing a small piece of liver from the edge of the most convenient lobe. The wound was closed with surgical clips, another incision was made at the shoulder region, and a muscle sample was cut from the triceps brachii. The sample was cleaned of fascia, and both the liver and muscle samples were cut in two. One portion of each was used for quantitative chemical analysis, the other for histochemical localization of glycogen.

Usually a small amount of arterial blood welled from the cut in the foreleg. One-tenth milliliter of this was drawn for blood sugar determination, and the cut was closed by sutures. If there was an insufficient amount of blood from this source, the femoral artery was exposed and divided and blood collected after quickly ligating the vessel so that too much blood

would not escape. The whole operation took from 10 to 15 minutes depending on the ease with which the blood sample was collected. Because the animals were hibernating, anaesthesia was not necessary.

After the operation was complete, the animal was fitted with cheek pouch and rectal iron-constantan thermocouples, as described previously (Lyman, '48), and then placed on a bed of cotton wool. The rise in cheek pouch and rectal temperature was recorded on a Micromax thermoelectric instrument.[2] Waking hibernators which showed an abnormally slow rise in body temperature were not included in the final data.

At a suitable time during the process of arousal the animal was killed by a blow on the head. The heart was quickly exposed and removed for glycogen determinations. A sample of mixed blood was then drawn from the pericardial cavity. A sample of liver was severed from an uninjured lobe, and lastly, a muscle sample was removed from the triceps brachii of the intact foreleg. This whole operation took, at most, 4 minutes. When the tissue samples were removed, two assistants immediately divided them, so that only a few seconds elapsed between the time the sample was taken and the time it was immersed in a suitable reagent. The tissue samples from control hamsters which were not hibernating were obtained in the same manner.

In one series of experiments the blood supply of the liver was cut off by ligation to discover if the hibernator could awaken without this source of glycogen. The animals were eviscerated in the manner described previously (Lyman and Chatfield, '50). The stomach and intestines were removed completely, but the liver was left within the body cavity. We have shown that this operation stops the blood flow to the liver. After evisceration, a liver sample was taken immediately and the abdominal cavity closed. The muscle and blood sample were then obtained as described above. In two of the eviscerated animals one lobe was separated from the rest

[2] Model S. Leeds Northrop Co., Philadelphia, Pa.

of the liver by means of an electric cautery knife and then left in the body cavity to determine the rate of anaerobic glycolysis in completely unattached liver tissue. When the experiment was terminated, a sample from the undamaged portion of the isolated piece and a sample from an uninjured lobe were taken for comparison. Evisceration added about 8 minutes to the sample-taking operation.

In the initial measurements of blood sugar, the blood was obtained by heart puncture, or by cutting the femoral artery of unanaesthetized, hibernating hamsters. One-tenth milliliter blood was analyzed for blood sugar using the Folin and Svedberg ('30) method. Later, when liver and muscle glycogen were also measured, the blood sugar was determined by the anthrone method of Morris ('48), using 0.5 ml of blood filtrate prepared by Haden's ('23) modification of the Folin-Wu technique. In a few cases the blood filtrate was colored, and these determinations have been omitted from the data. Comparative determinations of two samples of blood showed a maximum difference of 4% between the two methods.

The glycogen was obtained from the tissue samples by the method of Good, Kramer and Somogyi ('33), using 2.0 ml KOH and tissue weights varying from .0113 to .1783 gm for the liver, .0124 to .5032 gm for the muscle and .0524 to .3613 gm for the heart. The glycogen was precipitated with two volumes of alcohol, centrifuged and dissolved in water. Aliquots were taken for color development by the anthrone reaction of Morris. In both blood sugar and glycogen determinations involving the anthrone reagent the color was read in a Klett-Summerson colorimeter at 620 μ.

For histochemical localization of glycogen in liver and muscle, the tissues were fixed in chilled Rossman's fluid (9 parts saturated solution of picric acid in absolute alcohol, one part neutral formalin), dehydrated, and imbedded in paraffin (Deane, Nesbett and Hastings, '46). Sections of 5 μ were treated with periodic acid-leucofuchsin (Schiff) reagents for staining tissue polysaccharides (McManus, '48; Hotchkiss, '48). In order to determine that glycogen deposits were being

stained and to differentiate them from other tissue polysaccharides, the glycogen was removed from control sections by digestion in 1% malt diastase, pH 6.8, for 15 minutes at room temperature prior to staining (Lillie and Greco, '47).

RESULTS

Carbohydrate levels in the hibernating and non-hibernating states

A comparison of the blood sugar and the glycogen content of liver, skeletal muscle and heart in hibernating hamsters

TABLE 1

Average tissue carbohydrate levels in control hibernating and non-hibernating hamsters. The figures for the hibernating animals that were subsequently aroused (tables 2 and 3) are included here

CONDITION	BLOOD SUGAR			LIVER GLYCOGEN			MUSCLE GLYCOGEN			HEART GLYCOGEN		
	No. animals	Ave.	S.D.	No. animals	Ave.	S.D.	No. animals	Ave.	S.D.	No. animals	Ave.	S.D.
	mg %			*mg/gm*			*mg/gm*			*mg/gm*		
Hibernating	25	126	21.4	20	31.5	11.0	21	7.3	2.4	4	4.6	.8
Hibernating after fasting	2	143	..	4	30.3	4.9	4	7.4	1.5	4	8.7	.3
Awake in cold room	6	97	7.5	6	29.3	8.0	6	7.7	3.2	3	2.3	.8
Awake in warm room	6	113	18.8	5	36.8	14.4	6	5.7	2.5	4	1.7	.5

and in hamsters awake in the cold room or the warm room is given in table 1. As is to be expected in animals fed ad libitum, there is a great variation between individuals within each group. However, except for the glycogen content of the heart, there appears to be very little difference among the three groups. The awake animals, whether in cold or warm environments, are virtually identical. The hibernating hamsters exhibit a consistently higher level of heart glycogen and their blood sugar is somewhat higher than that of non-hibernators. The latter finding is of particular interest in

view of the fact that in all hibernating animals previously studied (Kayser, '50) the blood sugar has been significantly lower than in the awake animals.

The effect of the state of nutrition of animals in hibernation was tested by waking 4 hibernators and letting them re-enter hibernation without being allowed to eat. When they were killed two days later, it was found that there was still some food in the esophageal portion of their stomachs. The pyloric and fundic portions and the small intestines were filled with a mucous-like substance which we have found to be present in all hamsters during hibernation (see Bensley, '02, p. 138 for nomenclature of the parts of the stomach of Cricetus frumentarius). The caeca were filled with material, a condition which is also typical of normal hibernation. These animals must have been approaching the starvation level, however, for two other hibernators which had been denied food subsequently woke a second time, 4 to 5 days later, and then died within 24 hours after the second waking. The blood sugar and liver and muscle glycogen of the 4 animals which entered hibernation without eating were well within the normal ranges, as shown in table 1. The heart glycogen, however, was almost double that found in normal hibernators.

Carbohydrate changes during arousal from hibernation

In studying the changes in carbohydrates during the process of waking from hibernation, we attempted to obtain samples of blood, liver and skeletal muscle at the start, in the middle, and at the end of the waking process. With one exception (see figs. 1, 2 and 3), we found that such a procedure was too traumatic and that the hamster started to chill after the second sample had been taken. It was therefore necessary to limit the study to two samples of each tissue from a single waking animal.

It is of some incidental interest that the cause of chilling after the second sample had been taken appeared to be loss of vasomotor control. It has been emphasized previously

(Lyman and Chatfield, '50; Chatfield and Lyman, '50) that an essential part of the waking process is a differential vasoconstriction, so that the anterior part of the animal warms much more quickly than does the posterior portion. Apparently this mechanism was disturbed when the second tissue samples were taken, because immediately thereafter the rectal temperature rose to the level of the cheek pouch temperature. We have never observed a hamster which is capable of waking from hibernation if the loss of vasomotor control has necessitated warming the whole body at once.

During the first 50 to 70 minutes of the waking process there was little change in the level of stored carbohydrate compared to that found in the hibernating animal. Thus, in a series of 6 of these animals the blood sugar averaged 126

TABLE 2

Diminution in glycogen in liver and skeletal muscle during arousal from hibernation, expressed as averages for 8 animals

	START OF WAKING	END OF WAKING
Liver glycogen,	ave. 37.0 (S.D., 10.9) mg/gm	13.5 (S.D., 9.4) mg/gm
Muscle glycogen,	ave. 6.3 (S.D., 1.9) mg/gm	2.1 (S.D., 1.5) mg/gm

(S.D. 27) mg %, the liver glycogen 35.5 (S.D. 12.4) mg/gm and the muscle glycogen 7.6 (S.D. 2.3) mg/gm.

It was during the last two or more hours, when body temperature was rising rapidly, that the significant changes in carbohydrate levels took place. In spite of the great variability in liver and muscle glycogen at the start of the waking process, both of these sources of stored energy were invariably much lower by the time the animal had warmed itself to 37°C. This is shown above in table 2 for a series of 8 animals.

One hamster, which is not included in the above table, is of especial interest. When the first sample was taken, it was noted that the liver appeared to be unusually fatty, and this was later verified by histological examination. The liver glycogen at the start of waking was 7.1 mg/gm, which is ab-

normally low. At the end of a normal waking period there was only a trace of glycogen in the liver (.11 mg/gm) showing that the animal had totally exhausted this source of energy. The muscle glycogen dropped from 4.0 to 2.6 mg/gm, a change well within the range established for normal arousal from hibernation.

Direct comparison, in a single animal, of heart muscle during hibernation and after waking was, of course, impossible. However, the average value for glycogen in 4 hibernators was 4.6 mg/gm (table 1), while the average value for 11 animals just after waking was 2.8 (S.D. .8) mg/gm, somewhat higher than that found in normal awake animals. It is therefore apparent that all three of these stores of carbohydrate, the liver, skeletal muscle and cardiac muscle, are depleted markedly during the waking process.

There were no definite trends in blood sugar changes from the beginning to the end of the waking process. Usually only a slight rise or fall in blood sugar occurred, well within the limits established for awake animals and hibernators (110 to 150 mg %), although in two cases out of 10, the final blood sugar rose to over 300 mg % and one other animal had a final blood sugar of 195 mg %. The blood sugar never dropped to hypoglycemic levels during the waking process, for the lowest terminal blood sugar was 95 mg %.

Carbohydrate changes during arousal with liver isolated. In view of the apparent role of the liver as a source of energy during arousal, it seemed pertinent to examine further the observed fact that hamsters can wake from hibernation with the blood supply to the liver completely cut off (Lyman and Chatfield, '50). To this end, a series of 4 hamsters were eviscerated during hibernation and samples of blood, muscle and liver removed in the usual manner. In the process of evisceration the blood supply to the liver is cut off completely by ligation. Although in the previously described evisceration experiments (Lyman and Chatfield, '50) the highest recorded cheek pouch temperature was 32°C., in the present experiments the cheek pouch temperature rose to the normal level

of 37°C. As is the usual case in eviscerated hibernators, the rectal temperature rose only slightly. When the cheek pouch temperature reached 37°C., the hamsters did not present the normal appearance of awakened hibernators, for they moved with difficulty and felt stiff to the touch.

A comparison of the blood sugar of these animals in hibernation with the final blood sugar when they had wakened showed a drop from an average of 129 (S.D. 9.0) to the hypoglycemic average level of 44 (S.D. 9.1) mg %. Muscle glycogen dropped from 9.6 (S.D. 0.3), to 5.6 (S.D. 3.2) mg/gm, a diminution similar to that found in normal waking hibernators. The liver, which was isolated from its blood supply

TABLE 3

Diminution of glycogen in the livers of two animals during arousal, after the blood supply to the liver had been cut off by ligation and after one lobe had been severed completely but left in the body cavity

ANIMAL	LIVER GLYCOGEN WHILE HIBERNATING	LIGATED LIVER AFTER WAKING	COMPLETELY ISOLATED LIVER AFTER WAKING
652	18.7 mg/gm	7.3 mg/gm	7.2 mg/gm
677	27.6 mg/gm	3.1 mg/gm	6.8 mg/gm

by ligation, showed a decline from an average of 31.0 (S.D. 10.8) to 9.3 (S.D. 5.0) mg/gm. That this decline in liver glycogen was due to anaerobic glycolysis was demonstrated in two of the animals by comparing the glycogen content of the intact liver isolated from its blood supply with a piece of liver lying completely free in the body cavity, as is shown in table 3.

In three eviscerated animals which were killed as soon as the body temperature reached 37°C., the heart glycogen was slightly higher than that found in the uneviscerated waking hibernators (3.5 compared to 2.8 mg/gm). The 4th eviscerated animal was killed one hour and 40 minutes after the peak temperature was reached, during which time the cheek pouch temperature declined slowly. The glycogen in the heart of

this animal was .35 mg/gm, which low figure probably reflects the exhaustion of stored glycogen.

Histochemical localization of tissue glycogen

The sites of stored glycogen and the changes in its amount and distribution during the process of arousal were demonstrated by the periodic acid-Schiff procedure in tissue sections. In preliminary experiments, tissue samples from each lobe of the liver and from different parts of single lobes in both hibernating and awake animals were compared. Glycogen concentration and distribution were found to be uniform throughout the liver in each individual, hence only single samples were taken in subsequent experiments. Estimations of relative amounts of glycogen made by microscopic inspection of the sections showed good correlation with the chemical determinations in liver and in heart, so that the histochemical preparations served as controls for the chemical determinations.

Liver glycogen varied in amount in different individuals among the hibernators and the awake animals but, as was shown by the chemical determinations, there was no pronounced difference in glycogen concentration between these two groups of animals. On the other hand, there was a definite difference in the distribution of the glycogen. In 7 out of 8 awake animals in either cold or warm rooms the greatest concentration of glycogen was found in the central zone of the hepatic lobules, whereas in 4 fasting hibernators and in 17 out of 22 normal hibernators the glycogen was uniformly distributed throughout the lobules (fig. 1) and in three there was a somewhat heavier deposit of glycogen in the portal zone of the lobules. It is to be expected that considerable variation in the distribution of glycogen in the liver might occur in awake animals that are fed ad libitum. All the animals described here were killed at the same time of day, so the similarity of glycogen distribution in their livers may represent a stage in a normal diurnal cycle of glycogen deposition and utilization (Deane, '44). In the hibernating ani-

mals the rate of metabolism is so low there presumably is very little turnover of stored foodstuffs and the more uniform distribution of glycogen within the liver lobule and the similarity of this distribution in a large number of animals are not surprising.

During arousal from hibernation a diminution in the amount of glycogen occurred throughout the hepatic lobules (figs. 1, 2, 3). In 7 out of 9 animals that were allowed to awaken completely, however, there was a distinctly greater loss of glycogen from the central zone than from the portal zone of the lobules. Under other conditions of glycogenolysis, in the livers of mice and of rats, Deane and co-workers (Deane, '44; Deane, Nesbett and Hastings, '46) found that glycogen loss occurred first from the central zone of the hepatic lobule and that only in the final stages of liver glycogen depletion did the decline in glycogen concentration proceed more rapidly in the portal zone than in the central zone.

Glycogen in skeletal muscle usually was precipitated in close proximity to the Z membrane which transects the muscle fiber or, when glycogen was fairly abundant, it appeared in rows between the fibrils. We were unable to predict the glycogen content of muscle by inspection of histochemical preparations, but a diminution in the amount of glycogen in a single individual during arousal always was evident.

The glycogen of the heart is extremely labile (Illingsworth and Russell, '51) and in spite of our attempts to fix it as rapidly as possible in a chilled fixing fluid which minimized the migration of glycogen in liver cells (Deane, Nesbett and Hastings, '46) all glycogen in all cardiac muscle fibers drifted to one side of the cell. The glycogen content of this tissue, therefore, could be evaluated only from cross sections of the fibers or from longitudinal sections which included the compacted mass of glycogen. In spite of this difficulty, good correlation was obtained between chemical determinations of glycogen concentration and estimations of relative glycogen content from tissue sections. Heart glycogen was most abundant in fasting hibernators (fig. 6), distinctly less abundant in non-

fasting hibernators (fig. 5) and least abundant in awake controls and in aroused animals (fig. 4). Thus, the histochemical preparations provided visual confirmation of the diminution of glycogen in the liver, skeletal muscle and heart during the process of arousal from hibernation.

DISCUSSION

Carbohydrate levels in hibernating and non-hibernating states. It is apparent that the blood sugar and the liver and muscle glycogen of the hibernating hamster are essentially within the normal range when compared to the awake animal. The heart glycogen level, on the other hand, is significantly higher during the hibernating state. All of these factors show wide fluctuations both in the awake and the hibernating state, and without a very large series of measurements it is not possible to determine whether there is a greater variation when the animal is hibernating or when it is awake.

It is unique that the blood sugar in the hibernating hamster actually averages slightly higher than in the awake animal. Since the work of Dubois (1896), whose figures now appear questionable, it has been generally agreed that the blood sugar of hibernating mammals is lower than when the animals are not hibernating (Suomalainen, '35; Kayser, '50). Dworkin and Finney ('27) believed that hypoglycemia was an essential prerequisite of hibernation and they produced a hypothermic state in the woodchuck *(Marmota)* by injecting large amounts of insulin. Suomalainen ('39) obtained a state similar to hibernation by injecting hedgehogs *(Erinaceus)* with a mixture of magnesium and insulin. Other workers, however, have questioned the validity of Dworkin and Finney's results (Dische et al., '31; Lyman, R. A., '43) and have shown (Feinschmidt and Ferdmann, '32; Musacchia and Wilber, '51) that ground squirrels *(Citellus)* often hibernate when the blood sugar is very near normal.

The high blood sugar in hibernating hamsters when compared with other hibernating rodents may be explained by a difference in habits. Many rodents, including the woodchuck

(Marmota), ground squirrel *(Citellus)* and doormouse *(Myoxis)* become very fat in the fall and hibernate for long uninterrupted periods. Kayser ('40) reports a doormouse *(Myoxis)* which hibernated continuously for 110 days. On the other hand, at least in the laboratory, the hamster tends to lose weight before entering hibernation (Lyman, '48). In a series of over 600 animals in this laboratory the average period of continuous hibernation has been less than 10 days. After a few days in hibernation the hamster awakens, eats and drinks a little and returns to the hibernating state.

Although the life history of hibernating animals is not known well enough to draw definite conclusions, it appears likely that mammals which become obese before hibernating depend on their stored fat for energy during hibernation and tend to remain in the hibernating state for long periods. Mammals such as the hamster, which store food, do not need to build up fat as a source of energy, but probably waken from hibernation more often in order to restore their energy sources by eating. If this hypothesis is correct, one would expect that the blood sugar of animals which hibernate for long periods would tend to become lower as the animal continued in the hibernating state. Feinschmidt and Ferdmann ('32) in a small series of ground squirrels *(Citellus)* have indicated that this does occur. Our determinations with the hamsters show no indication of a drop in blood sugar in animals which have hibernated continuously for as much as 7 days, but the variation in the blood sugars of normal hibernators is so great that only a striking decline would be significant.

The 4 animals which had been deprived of their food after waking from hibernation exhibited no lower than average blood sugar or liver and muscle glycogen when they returned to the hibernating state, and heart glycogen was higher than normal. Since there was still food in the esophageal portion of their stomachs, it would seem that they had not reached a starvation level. However, the death of the animals which awakened a second time after being deprived of food shows

that it is essential for hibernating hamsters to eat during the short periods when they are awake.

Whatever the reason for high blood sugar in hibernating hamsters, it is apparent that the premise that low blood sugar is a prerequisite to hibernation finds an exception in this rodent.

It seems significant that the glycogen of the heart is higher in the hibernating state than when the hamsters are not hibernating, for we have previously emphasized (Lyman and Chatfield, '50; Chatfield and Lyman, '50) that the heart is of primary importance during the waking process and that there is an increase in heart rate before any measurable rise of body temperature. With a high glycogen content, the heart of the hibernating hamster is capable of increasing its work load at the start of the waking process without depending on some exogenous source for energy.

Carbohydrate changes during arousal from hibernation. Comparative measurements of liver and muscle glycogen in individual animals (table 2) show that no matter what the level of liver and muscle glycogen at the start of waking from hibernation may be, both of these stores of energy are lower when the waking process is complete. A comparison of the heart glycogen of the normal hibernator with that of animals newly aroused from hibernation indicates that there is also a decline in glycogen in this organ. The depletion of glycogen takes place during the last two or three hours of the waking process, when the animal is shivering, the heart rate is increasing, and body temperature is rising rapidly.

In spite of the great expenditure of energy during the process of arousal, the blood sugar is usually maintained at normal levels and never drops to a dangerously low level. In a few cases an abnormally high blood sugar is found in a newly awakened hamster. We were unable to correlate the appearance of this hyperglycemia with any particular condition of the liver glycogen or with the speed of the awakening process. Gemmill ('42) points out that a rise in blood sugar often occurs in athletes during severe but not exhaustive

exercise. Arousal from hibernation could perhaps be classified in this category (Lyman, '48).

By isolating the liver of animals while they are hibernating, it is possible to show that the hamster is still capable of warming the anterior portion of its body to homeothermic levels. When the liver is ligated, however, a profound hypoglycemia occurs when the hibernator awakens. This demonstrates that the liver is a necessary source of glycogen in the normal process of arousal from hibernation.

It is well established that the glycogen of muscle is utilized directly as a source of energy, but that it cannot serve directly as a source of blood glucose (West and Todd, '51). This is confirmed in the experiments reported here. In the eviscerated animals, in spite of the pronounced hypoglycemia which developed during arousal, the drop in muscle glycogen was no greater than that in normally awakening hamsters. The hamster mentioned above that had an unusually fatty liver emphasizes this point. The liver contained little glycogen at the beginning of arousal, and although this was reduced to only a trace by the time the animal was fully awake, the drop in muscle glycogen was no greater than in other aroused animals. The loss of glycogen in skeletal muscle apparently represents that utilized, in part at least, by the muscle itself when the animal is shivering and making gross movements.

The diminution in heart glycogen during arousal, as suggested above, is probably the result of its utilization by the cardiac fibers during the rapid increase in the activity of the heart, especially during early stages of arousal. Heart glycogen is high in hibernating hamsters and falls to normal levels during normal arousal. The animals which awakened from hibernation and were deprived of food showed exceptionally large amounts of glycogen in the heart. Macleod and Prendergast ('29) have reported that there is an increase in glycogen in the heart of rats under conditions in which glycogen stores in the rest of the body become abnormally low, and it is possible that the same mechanism is operating here.

Viewing the process of arousal from hibernation as a whole, we have previously suggested on the basis of other evidence that this process was actuated and mediated by the sympathetico-adrenal system (Lyman and Chatfield, '50; Chatfield and Lyman, '50). The classical studies of Cannon ('25) and his co-workers have shown that the sympathetico-adrenal system is of prime importance in the mobilization of carbohydrate during times of physiological stress. The integrated utilization of carbohydrates during the process of arousal gives added credence to the importance of this system during this organized and energetic phase of the hibernating cycle.

SUMMARY

Blood sugar and liver, heart and skeletal muscle glycogen of hamsters in the hibernating and non-hibernating states and during arousal from hibernation were studied by microchemical and histochemical methods.

In spite of wide individual variations in each group, hibernating hamsters and non-hibernating hamsters in either cold or warm environments exhibited no significant differences in liver and muscle glycogen. The hibernators, however, were found to have significantly higher levels of heart glycogen and, unlike other hibernating mammals which have been studied, they have a somewhat higher blood sugar than the non-hibernating hamsters. This indicates that a low blood sugar is not a prerequisite of hibernation, as has been previously stated. In animals that were prevented from eating before they entered hibernation, the blood sugar and the liver and muscle glycogen were well within normal ranges for hibernators, but the heart glycogen was almost double that found in normal, non-fasting hibernators.

During arousal from hibernation there was little change in tissue glycogen levels in the first hour, but subsequently, when body temperature rose rapidly, there was a marked loss of glycogen from liver, muscle and heart. Glycogen loss occurred uniformly throughout all cardiac and skeletal muscle fibers. In the liver some glycogen diminution took place throughout

the hepatic lobule, but the greatest loss occurred in the central zone of the lobule.

There were no definite trends in blood sugar changes from the beginning to the end of the waking process. There was usually only a slight rise or fall in blood sugar, well within the limits established for hibernating animals. However, in hamsters in which the blood supply to the liver was cut off by ligation at the beginning of waking, the blood sugar dropped to hypoglycemic levels by the time that the anterior portion of the animal had warmed. The change in muscle glycogen was similar to that found in normal waking hibernators.

It would appear from these results that the liver is the source of glycogen necessary for the maintenance of the blood sugar level during the normal process of arousal from hibernation. The diminution in heart and muscle glycogen during arousal probably represents that utilized directly by these tissues during the period when there is an increase in heart rate, when the animal is shivering and when movement begins. The mobilization of carbohydrates again emphasizes the importance of the sympathetico-adrenal system during the process of arousal from hibernation.

LITERATURE CITED

Bensley, R. R. 1902 The cardiac glands of mammals. Am. J. Anat., *2:* 105–156.

Cannon, W. B. 1925 Bodily changes in pain, hunger, fear and rage. D. Appleton & Co., New York and London.

Chatfield, P. O., and C. P. Lyman 1950 Circulatory changes during process of arousal in the hibernating hamster. Am. J. Physiol., *163:* 566–574.

Deane, H. W. 1944 A cytological study of the diurnal cycle of the liver of the mouse in relation to storage and secretion. Anat. Rec., *88:* 39–65.

Deane, H. W., F. B. Nesbett and A. B. Hastings 1946 Improved fixation for histological demonstration of glycogen and comparison with chemical determination in the liver. Proc. Soc. Exp. Biol. and Med., *63:* 401–406.

Dische, Z., W. Fleischmann and E. Trevani 1931 Zur Frage des Zusammenhanges zwischen Winterschlaf und Hypoglykämie. Pflügers Arch. f.d. ges. Physiol., *227:* 235–238.

Dubois, R. 1896 Physiologie comparée de la marmotte. Annales de l'Université de Lyon, 1–268.

Dworkin, S., and W. H. Finney 1927 Artificial hibernation in the woodchuck (Arctomys monax). Am. J. Physiol., *LXXX:* 75–81.

FEINSCHMIDT, O., AND D. FERDMANN 1932 Beiträge zur Biochemie des Winterschlafs. Biochem. Zeitschr., *248:* 107–114.

FERDMANN, D., AND O. FEINSCHMIDT 1932 Der Winterschlaf. Ergebnisse der Biologie, *8:* 1–74.

FOLIN, O., AND A. SVEDBERG 1930 Micromethods for the determination of non-protein nitrogen, urea, uric acid and sugar in unlaked blood. J. Biol. Chem., *88:* 85–96.

GEMMILL, CHALMERS L. 1942 The fuel for muscular exercise. Physiol. Rev., *22:* 32–53.

GOOD, C. A., H. KRAMER AND M. SOMOGOYI 1933 The determination of glycogen. J. Biol. Chem., *100:* 485–491.

HAYDEN, R. L. 1923 A modification of the Folin-Wu method for making protein-free blood filtrates. J. Biol. Chem., *56:* 469–471.

HOTCHKISS, R. D. 1948 A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. Arch. Biochem., *16:* 131–141.

ILLINGWORTH, B. A., AND J. A. RUSSELL 1951 The effects of growth hormone on glycogen in tissues of the rat. Endocrinol., *48:* 423–434.

KAYSER, C. 1940 Exchanges respiratoires des hibernants à l'état de sommeil hibernal. Ann. de Physiol., *16:* 129–221.

———— 1950 Le sommeil hibernal. Biol. Rev., *25:* 255–282.

LILLIE, R. D., AND J. GRECO 1947 Malt diastase and ptyalin in place of saliva in the identification of glycogen. Stain Tech., *22:* 67–70.

LYMAN, C. P. 1948 The oxygen consumption and temperature regulation of hibernating hamsters. J. Exp. Zool., *109:* 55–78.

LYMAN, C. P., AND P. O. CHATFIELD 1950 Mechanisms of arousal in the hibernating hamster. J. Exp. Zool., *114:* 491–516.

LYMAN, R. A. 1943 The blood sugar concentration in active and hibernating ground squirrels. J. Mammal., *24:* 467–474.

MACLEOD, J. J. R., AND D. J. PRENDERGAST 1929 Glycogen in the heart and skeletal muscles in starved and well-fed animals. Trans. Roy. Soc. Can., *15* (V): 37–40.

MCMANUS, J. F. A 1948 Histological and histochemical uses of periodic acid. Stain Tech., *23:* 99–108.

MORRIS, D. L. 1948 Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science, *107:* 254–255.

MUSACCHIA, X. J., AND C. G. WILBER 1951 Studies on the biochemistry of Citellus. Fed. Proc., *10:* 95.

SUOMALAINEN, P. 1935 Über den Winterschlaf des Igels. Ann. Academia Scientiarum Fennicae, Ser. A. Tom. XLV *2:* 1–115.

SUOMALAINEN, P. 1939 Hibernation of the hedgehog VI. Serum magnesium and calcium: Artificial hibernation. Ann. Academia Scientiarum Fennicae, Ser. A. Tom LIII *7:* 1–16.

WEINLAND, E., AND MAX RIEHL 1908 Über das verhalten des Glykogens beim heterothermen tier. Zschr. f. Biol., *50:* 75–92.

WEST, E. S., AND W. R. TODD 1951 Textbook of Biochemistry. The Macmillan Company, New York, N. Y.

PLATE

PLATE 1

EXPLANATION OF FIGURES

Photomicrographs of 5 μ sections fixed in Rossman's fluid and stained with periodic acid-Schiff for the demonstration of glycogen. A Wratten B green filter was used to accentuate the fuchsin color and all pictures were printed to produce comparable color intensity.

1 Liver of a hibernating hamster fixed 43 minutes after the beginning of arousal. In this and in the two following figures the portal zone of the hepatic lobule is at the left, the central zone at the right. A large amount of glycogen is uniformly distributed throughout the lobule. Chemical determination of glycogen content: 40.9 mg/gm. × 125.

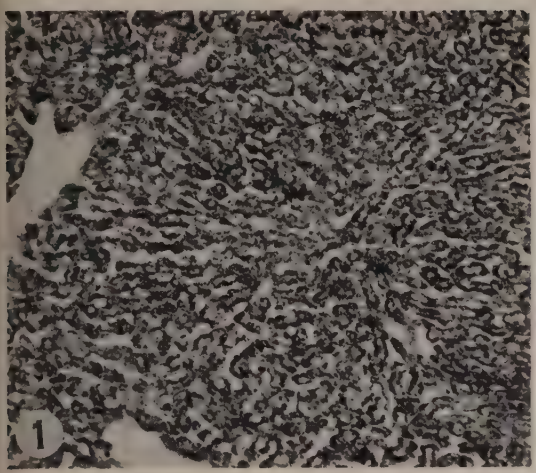
2 Liver of the same animal fixed 34 minutes later when cheek pouch temperature had risen to 11°C. Glycogen loss is evident throughout the lobule but is more pronounced in the central zone. Chemical determination of glycogen content: 33.2 mg/gm. × 125.

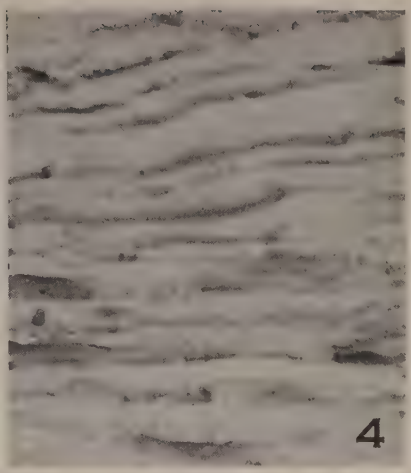
3 Liver of the same animal fixed when arousal was complete, 130 minutes after the beginning of waking. Glycogen concentration has diminished further throughout the lobule. It is abundant only in those cells contiguous to the portal canal, almost absent in the middle zone of the lobule, and irregularly distributed in a mosaic pattern in the central zone. Chemical determination of glycogen content: 8.4 mg/gm. ×125.

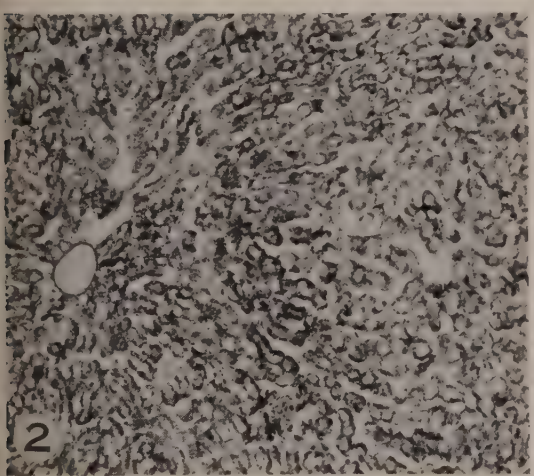
4 Cardiac muscle from a control hamster maintained in a warm environment. A very small amount of glycogen can be detected as fine dark granules deposited along one side of each muscle fiber. Chemical determination of glycogen content: 2.13 mg/gm. × 300.

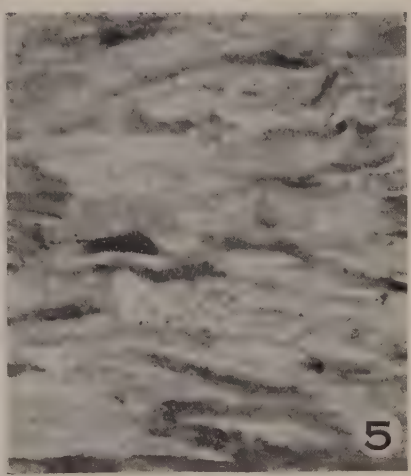
5 Cardiac muscle from a normal hibernating hamster. Glycogen is much more abundant than in control above. Chemical determination of glycogen content: 4.04 mg/gm. × 300.

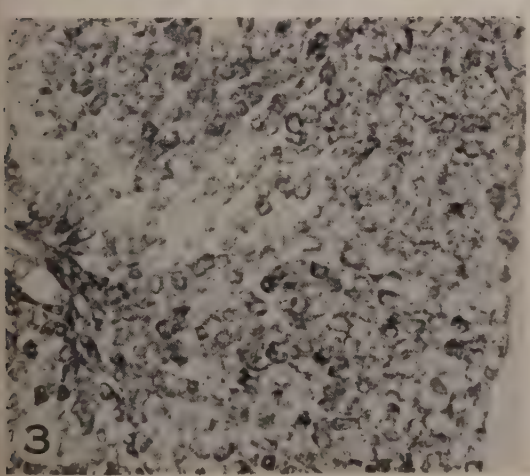
6 Cardiac muscle from a hibernating hamster which had been deprived of food. A heavy accumulation of glycogen is present throughout all muscle fibers. Chemical determination of glycogen content: 8.56 mg/gm. × 300.


1


4


2


5


3


6

PHOSPHOPROTEIN PHOSPHATASE ACTIVITY IN NORMAL, HAPLOID AND HYBRID AMPHIBIAN DEVELOPMENT [1, 2]

LISELOTTE MEZGER-FREED
Department of Zoology, Columbia University, New York City, N. Y.

SIX FIGURES

The observation that food sources for embryos and young (i.e., yolk and milk) contain a high proportion of phosphoproteins compared to already differentiated tissues (Needham, '31) indicates that an investigation of the phosphoprotein metabolism and distribution would provide a clue to some developmental processes. In 1946 Harris discovered in frogs' eggs an enzyme, phosphoprotein phosphatase (PPPase), which separates phosphate from protein without previous hydrolysis. Barth and Jaeger ('50, '51) found that the enzyme may be active in a transfer system having ATP as one of its components; it was suggested that phosphoprotein may be not only a source of inorganic phosphate and of protein building material, but perhaps also a store of high-energy phosphate bonds.

An investigation of the PPPase activity during development of the normal, haploid and hybrid *Rana pipiens* embryos, as well as the distribution of PPPase activity in the gastrula of the normal and hybrid embryo, was undertaken as a basis for further study of the enzyme. Enzyme activity at gastrulation would be of special interest since PPPase may well be active in the morphological changes occurring at

[1] This work was supported in part by a research grant from the Division of Research Grants of the National Institutes of Health, Public Health Service.

[2] A dissertation presented to the faculty of the Department of Zoology of Columbia University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

this stage. The hybrid *Rana pipiens* ♀ × *Rana sylvatica* ♂, which does not gastrulate (Moore, '46), would be expected to have a different PPPase activity from the normal embryo if the enzyme activity is correlated with gastrulation.

The activity was measured as the amount of phosphate phosphorus released from endogenous substrate in a brei per 10 minutes per embryo or per dry weight unit. The assumption is made that any differences in enzyme activity represent at least potential differences in the living embryo. The activity was determined in breis of normal *Rana pipiens* embryos from stages 1–25, in haploid embryos to stage 23 and in hybrids to stage 11, as well as in the various regions of the normal and hybrid gastrulae at stage 10.

An increased activity before dorsal lip formation was observed in all three types of embryos. After gastrulation in the normal and haploid embryos, the activity resumes its former level until the neural-tube stage, after which it decreases steadily. Differences between haploids and normals, if any, are too small to be detected in the number of experiments performed. However, the hybrid embryo has a shorter period of increased activity than the normal one. A further difference is evident upon analysis of the parts of the gastrula. In the normal embryo the regions above and below the dorsal lip have an equal PPPase activity, whereas in the hybrid the activity above the dorsal lip is significantly lower than the activity below the dorsal lip.

METHODS

Obtaining of embryos. The method used for obtaining eggs is outlined in Barth ('46). Fertility was better than 95% except in eggs available during the summer months, when it was as low as 70%. For hybrids, eggs from the same female were stripped into two suspensions, one of sylvatica sperm, the other of pipiens sperm; the latter provided a normal control for the hybrid.

The method of Rugh ('39) was used to obtain the gynogenetic haploids. One testis of a frog was placed intact in

10% Ringer's in the icebox; the other testis was laid on Ringer soaked cotton in a celluloid dish with cover. Except in one case, the dish was in an ice-bath for the whole period of exposure to radiation. A dosage of 70,000 r, shown by Rugh ('39) and Briggs ('51) to produce haploids, was given; the testis was exposed to 75.7 r/minute for 92.5 minutes, the machine operating at 210 kilovolts and 15 milliamperes at a target distance of 20 cm. A .11 Cu and a .5 Al filter were used to absorb long x rays. After exposure, the testis was placed in a vial of 10% Ringers on ice until ready for use, which was within the hour. Two separate sperm suspensions, one of irradiated, the other of control sperm, were made a few minutes apart and eggs from the same female stripped into both. The summer eggs used were about 70% fertile with control sperm, but only 35% with irradiated sperm.

Sperm which has been irradiated with a total of 50,000 r or more, according to Rugh ('40), is able to activate the egg but make little or no genetic contribution; about 90% of the eggs so treated are haploids (Briggs, '51). Although no actual count was made in the present experiments, it was observed that very few individuals produced did not have symptoms of haploidy, i.e., shorter length, curved back, edema. Moreover, chromosome counts on stages 20–25 using an aceto-carmine tail-tip smear method (Ting, '50) showed that of 21 individuals examined from the 4 experiments, only one was diploid ($2n = 26$). This is assumed to be the one embryo of normal appearance among those counted.

Determination of enzyme activity in whole egg breis. Eggs of the desired stage (Shumway, '40) were taken from the 14°C. environment and placed in a stender dish in an ice-bath. Under the binocular microscope the jelly was removed from 15 eggs which were dropped into a graduated centrifuge tube. The solution was carefully brought to 3 ml with more 10% Ringers and poured into a glass Ten Broeck homogenizer in an ice-bath. After homogenization the brei was stirred by blowing through a pipette and 0.5 ml aliquots were immediately pipetted into the following solutions in a 26°C. water

bath; three tubes containing 0.5 ml each of 0.2M glycine buffer at pH 3.5, and two tubes containing in addition 0.5 ml of a solution of 10% trichloroacetic acid and 0.4% uranium acetate. The tubes were immediately mixed by shaking and after 10 minutes' incubation at 26°C., the trichloroacetic acid solution was also added to the three tubes not already containing it. The fact that the phosphate in the control tubes was negligible demonstrates that little or no enzyme activity takes place in the ice-bath and that addition of trichloroacetic acid stops the reaction almost immediately.

The pH of the buffer was checked in a Beckman pH meter both before and after the addition of brei. At a buffer pH of 3.5 addition of homogenate caused a deviation of less than 0.1 pH units.

In order to determine the inorganic phosphate released by the enzyme, a modification of the Fiske and Subbarow ('25) method was applied. After allowing the proteins to precipitate for at least half an hour, distilled water was added to 9 ml and the tubes centrifuged at low speed for 10 minutes. The clear supernatants were decanted into another series of centrifuge tubes and 1.2 ml molybdate plus 4N H_2SO_4 solution added. A cloudiness was produced which would eventually resolve itself into a light yellow precipitate. A second centrifugation was therefore performed, the supernatant decanted into calibrated Klett tubes and 0.4 ml reductant, aminonaphtholsulfonic acid, added as well as enough distilled water to bring the solution to 10 ml. Neutralization prior to this step was unnecessary because of the use of uranium acetate instead of a strong TCA solution. After 10 minutes' color development, the tubes were read in a Klett-Summerson colorimeter with a no. 66 filter. The instrument was set at zero with distilled water and the control subtracted from the experimental reading. Using a standard curve, values were then calculated to read in terms of micrograms P released per egg per 10 minutes.

Because the centrifugation after the addition of molybdate solution is a new step in the Fiske-Subbarow procedure, a

series of experiments was performed to determine whether or not significant amounts of phosphate are lost. The above procedure was followed using various amounts of active or inactive homogenate in combination with several concentrations of standard phosphate solution; the results showed that phosphate recovery was not significantly changed by the centrifugation.

Microchemical determination. The reference standard for the determination of enzyme activity for parts of the egg was dry weight instead of the whole egg itself. The determination of phosphate was again that of Fiske and Subbarrow, but scaled for from 0–4 μg phosphate P. The parts were taken from a ring-shaped piece of tissue extending through the animal and vegetal poles and through the dorsal lip. In this way samples of possible animal-vegetal and dorso-ventral gradients could be obtained.

The eggs were dissected according to the method of Sze ('52) in Holtfreter's solution at the beginning of gastrulation when pigment was just beginning to aggregate and the dorsal lip to form. After being excised with a tungsten needle, the ring of tissue was cut at the animal pole, laid flat and the resulting strip divided into 6 parts; the first two cuts were made at the edge of the tufts of yolk at the top and the bottom of the strip, the former cut at the dorsal lip. The middle piece was divided in half (at the animal pole) and each of the resulting two pieces cut in two. The 6 pieces were numbered D, 1, 2, 3, 4, V, starting from the region below the dorsal lip, thence to the animal pole and ending with V in the ventral yolk zone. Thus the vegetal pole is between parts D and V, the animal pole between 2 and 3. They were removed to corresponding dishes of Holtfreter's in an ice-bath with a wide-mouthed medicine dropper. About 15 eggs were dissected for each experiment.

Before homogenization, salts were removed by transferring the tissue through two changes of distilled water and into a 200 μl microcentrifuge tube in an ice-bath. About 150 μl water were added and the tissues homogenized by plunging a bead-

tipped stirring rod to the bottom of the tube several times. After stirring by blowing through the pipette, 50 µl aliquots were transferred into (a) a microtube containing 90 µl glycine buffer at pH 3.5 and (b) a microtube containing in addition to buffer 30 µl of the trichloroacetic acid and uranium acetate solution. After stirring and incubating for 10 minutes in a 26°C. water bath, the TCA solution was also added to the former tube. Pipetting was done with a micropipette (Barth and Sze, '51). A constant pressure delivery apparatus was used when pipetting homogenate.

After at least 30 minutes' precipitation, the tubes were centrifuged and 120 µl of the supernatant transferred to a new set of microtubes to which 40 µl molybdate solution were added. Again the solution was centrifuged and as much of the supernatant as could be removed with a micropipette put into calibrated micro-colorimeter tubes. Ten minutes after the addition of reductant the optical density was measured on a Coleman Junior spectrophotometer using a 660 mµ wavelength. The instrument was zeroed with a blank containing distilled water instead of homogenate. Final readings were obtained by subtracting control from experimental readings and converting to µg P by means of a standard curve.

The dry weight was found by weighing an aliquot of homogenate on a quartz microbalance (Sze, '52). Both the pipette used to deliver the homogenate for incubation and that for dry weight were carefully calibrated by weighing deliveries of known $KHC_8H_4O_4$ solutions which had been dried. With a knowledge of the exact volume and weight of the aliquot whose dry weight was determined, and the exact volume and phosphorus content of the aliquot whose extinction was measured, the µg P per µg of dry weight were calculated.

Several experiments were tried using ascorbic acid as an activator (Feinstein and Volk, '49). However, since it was found that the compound acted as a reductant, giving standard curves as accurate as those with aminonaphtholsulfonic acid, no further use was made of it.

RESULTS

Properties of PPPase

pH optimum. Glycine (HCl), acetate, succinate, veronal and glycine (NaOH) buffers were used to provide a range of from one to 11 pH units. Addition of homogenate caused an average pH change of less than 0.2 in pHs from one to 11 and one of less than 0.05 in the two to 5 pH range. It is

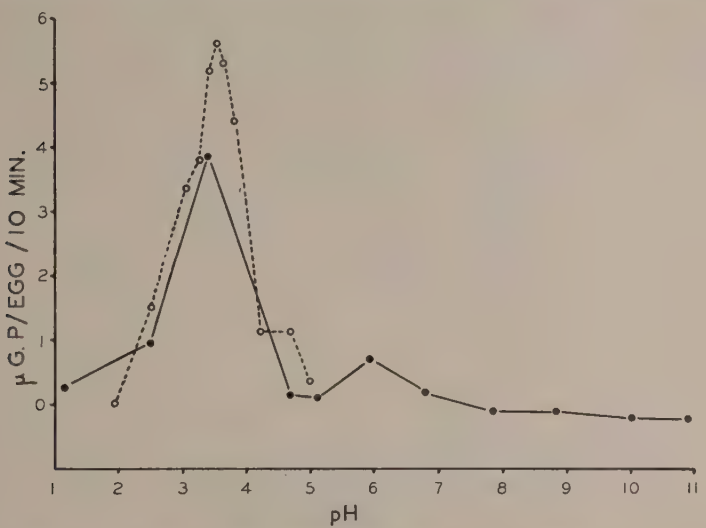


Fig. 1 The effect of pH on phosphoprotein phosphatase activity.

evident that comparatively little phosphate is split from endogenous substrate by any enzyme or enzymes other than the one active at pH 3.5. Why there is a difference of one and one-half pH units in the optimum found in the present study and that reported by Harris ('46) of pH 5.0 is not clear. If it is assumed that Harris' method releases only large, fully developed ovarian eggs, the wet weight is about 3 mg (Atlas, '38); the average amount of phosphate released in 10 minutes at 37°C. is about 400 mg per 100 gm wet weight or 12 μg per egg. This is about twice the amount released at a pH of 3.5 at 26°C. Although the difference in amount may be

due to the fact that citrate buffer dissolves yolk platelets more easily than glycine buffer, the difference in pH optima remains unexplained.

Temperature. Aliquots from a brei kept in an ice-bath were taken over a period totaling about 90 minutes and incubated at various temperatures. The loss in liberation of phosphate at the end of the 90 minutes was 10%, but this loss does not affect the conclusions. The optimum temperature for the

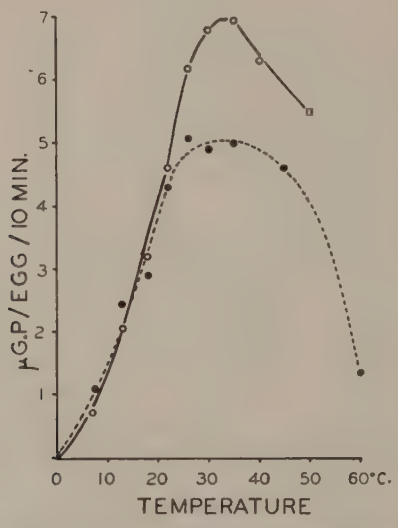


Fig. 2 The effect of temperature on phosphoprotein phosphatase activity.

reaction is between 30°C. and 35°C. The incubation temperature used in the experimental work was 26°C., which is close to the optimum and in addition is within the range of temperature tolerance for *Rana pipiens* embryos (Moore, '39). The curve obeys the Arrhenius equation from 13°C. to 26°C. giving a μ of about 15,000. The Q^{10} from 15°C. to 25°C. is 2.3.

Time. The rate of enzyme activity is constant for the first 10 minutes. The decrease in rate after this time is probably due to lack or non-availability of substrate as indicated by Barth and Jaeger's finding ('50b) that addition of an extract containing phosphoprotein substrate plus heat inactivated

enzyme to one containing both active enzyme and substrate increased the amount of phosphate released. Moreover, according to Kutsky ('50), there is a total of from 7 to 10 μg phosphoprotein phosphate in the egg.

Enzyme activity during normal development. Eggs from 10 females were analyzed for phosphoprotein phosphatase activity during development. The mean value for readings in the first 100 hours ranged from 1.7 μg P per egg to 7.0 μg P per egg for different batches of eggs, with a mean for all the batches of 4.4 μg P. That these differences are not correlated with the size of the egg is indicated by the experiments on parts

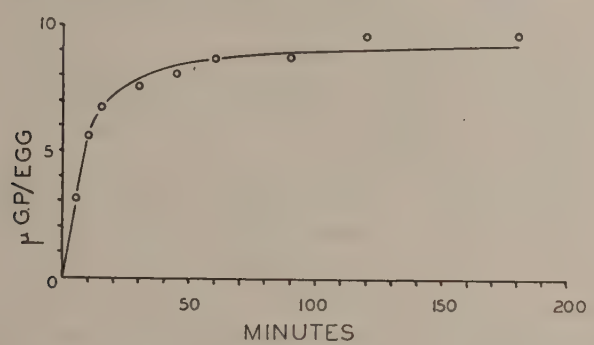


Fig. 3 The effect of length of incubation time on phosphoprotein phosphatase activity. (The curve was determined by means of the least squares method.)

of the gastrula in which there were also differences although determinations were made on a dry weight basis. In order to keep experiments with fewer readings from influencing the values disproportionately in any analysis which required averaging the readings from different experiments, the mean for the first 100 hours was brought to 4.4 for each batch by multiplying each reading by a factor $\frac{M_n}{M_x}$ or the common mean divided by the individual mean. Thus all readings within an experiment retained the same relative values.

From studying the individual patterns of PPPase activity throughout development, it is evident that there are few common trends. Activity may be fairly constant for the first

50 hours in one batch of eggs, or it may fluctuate, showing a decrease in activity in some and an increase in others. That these variations are not because of the method is indicated by several observations. Tests made on two groups of eggs from the same batch homogenized about 5 minutes apart show the same activity; few of the trends are indicated by only one point; a majority of the experiments have fluctuations at some time before gastrulation.

The data from the 10 experimental series were plotted in two ways, as a function of time and as a function of developmental stage. See figures 4 and 5. For the former the time scale was divided into blocks which became successively larger with time unless there were many readings, as at gastrulation, when smaller time units were used. The number of readings per block averaged 8 and ranged from 6 to 12. The average reading was then plotted against the average time at which the reading was taken to give the curve in figure 4. Assuming that the readings are normally distributed in each time block, the probability, p, that in two time blocks having the same mean the reading of one is greater than that of the other is one-half. In a series of experiments the probability, P, that these two values, having the same mean, will show a difference of the same sign x times out of n trials is given by the binomial equation

$$P = \frac{n!}{x!\,(n-x)!}\, p^x (1-p)^{n-x}.$$

This test was used to analyze the results.

The decrease in activity between the unfertilized and the newly fertilized egg is not significant when compared with either the first three hours or the first 20. The decrease between 12 and 20 hours is likewise not significant. However, the activity at 20–30 hours ($P = 5\%$), and that at 45–60 hours is greater than that from 20–30 hours ($P = 0.4\%$). No significant difference is found between 45–60 and 60–80 hours but that between 45–60 and 90–100 is significant, so it can be considered that 45–60 $\geqq$ 60–80 $\geqq$ 90–110. The increase from 140–180 is not significant. Considering the period from 20–90

hours, which may be regarded as preparation for gastrulation plus gastrulation, the average activity is greater than that from 0–20 hours in every experiment. Summarizing, 1–20 $<$ 20–30 $<$ 45–60 $\geqq$ 60–80 $\geqq$ 90–110, which represents in stages 1–6 $<$ 7 $<$ 9–10 $\geqq$ 10–11 $\geqq$ 12. The peak of activity is somewhere between 45 and 60 hours or just before and during the formation of the dorsal lip. It is evident from the

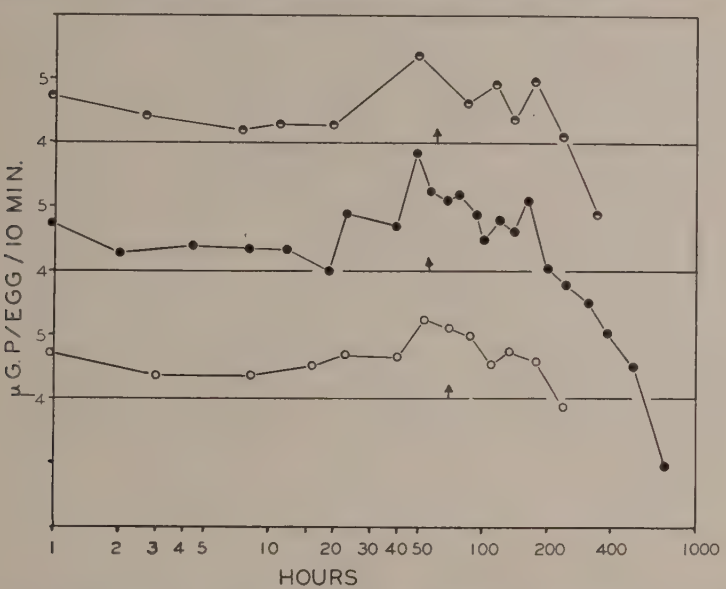


Fig. 4 Correlation of PPPase activity with hours of development at 14°C. The top figure is for haploid, the middle for normal and the bottom for hybrid embryos. The arrows mark the beginning of gastrulation.

data that after 180 hours (stage 16) the activity decreases at a nearly constant rate.

As would be expected, an analysis of the PPPase activity as a function of the stage of development gives results similar to those obtained above. See figure 5. The average number of readings per stage is 8 with a range of from 4 to 15 readings. It is again to be concluded that in the time preceding and during gastrulation the activity is higher than it is during the beginning of development; in every experiment the activity of stages 1–8 is less than that of stages 9–11. However,

in this analysis it is found that there is a decrease in activity after fertilization or between stages one and two ($P = 2\%$). Stages 9–10, stages 10–11 and stage 11 are all significantly higher than stages 1–8 ($P = 5\%$ for each). Other comparisons made revealed no significant differences except that stage 11 is greater than stage 12 ($P = 2\%$). Summarizing, stage $1 > 2\text{–}8 < 9 = 10 = 11 > 12$. Combining the results of

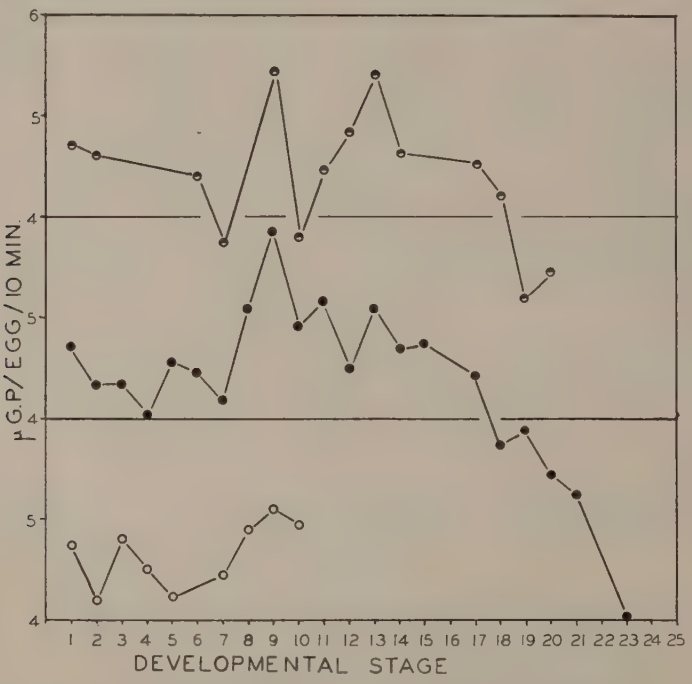


Fig. 5 Correlation of PPPase activity with stage of development. The top figure is for haploid, the middle for normal and the bottom for hybrid embryos.

the two analyses, it is found that there is a slight decrease in activity from before fertilization to first cleavage, a low point just before stage 7 when there is an increase; just before gastrulation begins at stage 9 the activity is at a peak which starts decreasing sometime during stage 10 and continues to decline to stage 12, where it has reached its initial activity. This level of activity is maintained through stage 15 and then declines steadily to stage 25.

An examination of the "corrected" data shows that there are two periods in development in which the range of the readings is less than 1 μg. One such interval is found at 54–62 hours (7 points), the period when gastrulation is beginning, and the other from 140–150 hours (7 points), the beginning of stage 14 when the neural folds are forming. Both of these are periods of development during which active and crucial movements are taking place.

In 4 of the experiments, 6 to 11 determinations were made in the first 12 hours of development (up to the 32-cell stage). In each case a decreased enzyme activity between three and 5 hours after fertilization of from two to 5 hours' duration was observed. This occurred before the first cleavage and was as much as a 75% change in activity. Another decrease was observed between 7 and 12 hours (stages 4–7). There is no obvious correlation of these changes with cleavage.

Enzyme activity during hybrid development. Individual developmental series comparing the hybrid (fig. 4) and normal embryos of the same female show that their PPPase activity follows a similar pattern in time, with the hybrid having a somewhat higher activity at one time, the normal at another. The differences are not usually large and do not seem to be regular. Although there are 4 degrees of freedom in the hybrid experiments rather than 6 to 8 as in the above experiments, the following can be said. Stage 9 has significantly more activity than stages 1–8 ($P = 6\%$) and stage 10 is definitely not significantly higher, although in the normal embryos from the same females it is ($P = 6\%$). It should be remarked that stage 10 is a difficult one for comparisons since activity seems to decrease, but this would favor the interpretation that the activity is lower in hybrids since they are slower to reach stage 10. This means that observations made on both types at the same hour would be made on a more advanced, therefore, lower stage 10 in normals. If time blocks are compared (fig. 5), the activity from 20–90 is not significantly higher than that from 1–20 hours, although it is higher at 40–90 hours ($P = 6\%$). From 75–100 hours, the

time at which the normal embryo is in stage 11, the PPPase activity is not higher than from 1–20 hours. The degrees of freedom would not permit small differences between normal and hybrid embryos to be detected and an analysis shows that there are no such differences for stages 7, 8, 9 and 10. The main conclusion to be reached is that there is not as prolonged a rise in activity in the hybrid as in the normal embryo, since it is only stage 9 which has an activity higher than that found at the beginning of development.

Enzyme activity during haploid development. In the haploids, as in the hybrids, the individual developmental curves follow closely in time and trends those of the normal embryos from the same female. See figures 4 and 5. The PPPase activity of the haploids is greater at stages 9–11 than at stages 1–8 and the activity from 20–90 hours greater than that at 1–20 hours ($P = 6\%$ for both). Any differences which may exist between haploids and normals are too small to be shown by three degrees of freedom and we may conclude that there are no significant differences in PPPase activity between the two.

Enzyme activity in the parts of the normal and hybrid gastrula. In view of the fact that at least the visual manifestation of the first part of gastrulation is a local one and that the hybrid does not continue this process, an analysis of PPPase activity in the different regions is indicated. The results of 5 experiments on the normal and on the hybrid are given in tables 1 and 2 and figure 6. An analysis of variance on a 5% level shows that in the normal embryo PPPase activity is as follows: $D > 2$, $1 > 3$ and $2 > 4$. Although $P = 20\%$ for the parts 1 and 2, part 1 can be considered greater than 2 since $D > 2$ and $D = 1$. Therefore, $D = 1 > 2 = 3 < 4 = V = D$. There is a vegetal-animal gradient with the regions (1 and 4) above the large yolky cells equalling the latter in activity. Parts 1 and 4 are equal, as are parts D and V, so that there is no ventral-dorsal gradient of activity.

In the hybrid, part 1 or the region just above the dorsal lip, is significantly lower than D, unlike the situation found

TABLE 1

The distribution of PPPase activity in the various regions of the normal egg in μg P/μg dry weight

EGG NO.	REGIONS					
	D	1	2	3	4	V
1	0.0032	0.0028	0.0028	0.0018	0.0032	0.0038
2	0.0039	0.0038	0.0018	0.0033	0.0030	0.0029
3	0.0018	0.0015	0.0018	0.0013	0.0020	0.0016
4	0.0024	0.0029	0.0026	0.0022	0.0033	0.0023
5	0.0022	0.0023	0.0015	0.0018	0.0017	0.0013
Mean	0.0027	0.0027	0.0021	0.0021	0.0026	0.0024

TABLE 2

The distribution of PPPase activity in the various regions of the hybrid egg in μg P/μg dry weight

EGG NO.	REGIONS					
	D	1	2	3	4	V
1	0.0039	0.0025	0.0022	*	0.0024	0.0036
2	0.0035	0.0019	0.0009	0.0023	0.0028	0.0022
3	0.0019	0.0018	0.0013	0.0016	0.0019	0.0015
4	0.0017	0.0013	0.0010	*	0.0020	0.0026
5	0.0021	0.0005	0.0018	0.0025	0.0024	0.0024
Mean	0.0026	0.0016	0.0014	0.0022	0.0023	0.0025

* Lost. The average per cent P for this region in the other experiments was used to calculate a mean.

in the normal gastrula. From the three determinations made on part 3, it appears to be higher than part 2 ($P = 15\%$ for two degrees of freedom); the significant difference between 2 and 4 and the fact that 3 and 4 are equal ($P = 35\%$) is further verification for the relationship $2 < 3$. The sequence is $D > 1 = 2 < 3 = 4 = V = D$. The lowest part of the gradient in the hybrid is between parts 1 and 2 rather than at the animal pole as in the normal gastrula. This difference gives the hybrid a ventral-dorsal gradient.

PPPase activity as related to other standards. Since we have available the Barth and Sze ('52a) figures on the rela-

tionship of total nitrogen, dipeptidase, cell number and extractable fraction to dry weight for the various regions of the *Rana pipiens* gastrula which were dissected, we are able to refer the PPPase activity to these standards. See figure 6. If total nitrogen as an index of total protein is used, it is found that the regions above the large yolk-filled cells (1 and 4) have a higher activity per unit of total nitrogen than the other

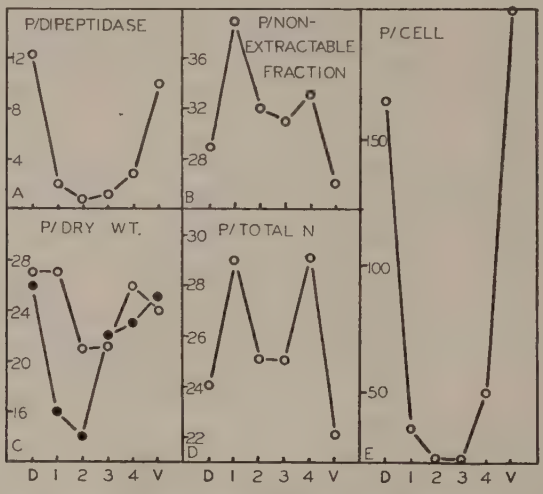


Fig. 6 PPPase activity in the regions of the early gastrula. ○ represents the normal gastrula, ● the hybrid. The abscissae represent the regions of the gastrula. See text. The ordinates are: (a) $x10^{-2}$ μg P/μl 0.05 N HCl for dipeptidase (b) $x10^{-4}$ μg P/μg dry weight for non-extractable fraction (c) $x10^{-4}$ μg P/μg dry weight for dry weight (d) $x10^{-3}$ μg P/μg total N (e) $x10^{-6}$ μg P/cell.

parts of the gastrula. A more significant comparison of the regions would be that made on the basis of enzyme activity per unit of yolk, or that fraction not extractable by 0.65% NaCl in phosphate buffer (Gregg and Løvtrup, '50), since yolk provides the substrate for this reaction. If the PPPase activity per per cent dry weight of non-extractable fraction is calculated, it appears that the high activity of the dorsal lip is to a large extent independent of the amount of substrate but that of the other regions, including the ventral counter-

part, part 4, is not. Further support for this statement comes from observation of the dissected gastrula; it is evident that the cells in region 4 are larger and yolkier than in region 1. Barth and Sze (to be published) found this also true when they determined the number of cells per μg of dry weight in the 6 regions; there are 77 cells in region 1 compared to 53 in region 4. In general it is true that the larger the cell, the higher is its PPPase activity as is evident from figure 6.

No comparable measurements of total N etc. have been made on the hybrid and since the lower activity found on the dorsal side of the embryo may be correlated with any of the substances used as reference standards, no comparison of normal and hybrid gastrulae was attempted.

Since it is known that the average dry weight of the *Rana pipiens* embryo is 1.3 mg, it is possible to compare the PPPase activity of the whole egg to that of the sum of its parts. A rough estimate would be given by taking the average enzyme activity per μg of the parts and comparing it to that of the whole egg. However, a more refined method would be to calculate directly the enzyme activity of the whole egg from that of its parts by using measurements by Barth and Sze (to be published) of the number of cells per μg in the 6 regions, and of the total number of cells in the vegetal hemisphere and in the dorsal half and ventral half of the animal hemisphere. The number of cells is then multiplied by the amount of P per cell. Using the average PPPase activity for parts D and V, the vegetal hemisphere has an activity of 2.1 μg P (11,000 $\times$ 0.000190); averaging the activities of parts 1 and 2, and of parts 3 and 4 respectively, the dorsal half of the animal hemisphere has an activity of 0.35 μg P (12,000 $\times$ 0.000029) and the ventral half also 0.35 μg P (9,600 $\times$ 0.000036). This gives a total of 2.8 μg P per egg, a value lower than the 5.0 μg per egg found with homogenates of the whole egg; the discrepancy is to be expected since in the latter experiments the regions with more substrate are combined with the regions

of higher enzyme activity; indeed, the greater enzyme activity per unit N is associated with the smaller yolk platelets, which are to be found in greatest concentration in the animal half of the egg (Panijel, '50). The values agree well with those of Barth and Jaeger ('51) on animal and vegetal halves of the gastrula minus the marginal zone; these were 2.0 μg P compared to the above 2.1 μg P for the vegetal zone and 0.4 μg P compared to 0.7 μg P for the animal hemisphere.

DISCUSSION

In any evaluation of the role of an enzyme based on determinations of its activity in homogenates, consideration must be given to the possible relationship of this activity to occurrences in the living organism. It is perhaps even more important in cases where there is not the reassurance which comes from correlating the activity with the emergence of a function in which other evidence indicates the enzyme plays a part. Since no additional substrate was supplied for the experiments reported here, the very enzymatic nature of the process releasing phosphate should be questioned. However, the pH optimum, the temperature optimum, the Q^{10}, all leave little doubt that this is an enzymatic reaction. That it attacks phosphoprotein, Harris demonstrated by showing that the increase in inorganic phosphate could be accounted for by the decrease in protein phosphate; furthermore, the enzyme will release phosphate from another phosphoprotein, casein. The omission of additional substrate has at least a theoretical advantage, however, since formation of product is dependent only on the amount of enzyme, substrate and cofactors present in the normal organism or in the living condition.

The nature of the change in enzyme systems upon homogenization is difficult to investigate experimentally. Certainly, it is evident that the release of 10 μg P per egg per hour in a brei does not normally occur in the living egg; according to Kutsky ('50) 7–10 μg is the total amount of phosphoprotein phosphate in *Rana pipiens* eggs. The present experiments give no evidence as to whether the acceleration is because of

the breakdown of an organization separating the enzyme and substrate, as Spiegelman and Steinbach believed was true in the case of greater oxygen consumption in breis ('45), or whether there are chemical changes in the brei conducive to enzyme activity. However, it is safe at least to assume that when under identical experimental conditions there is a change in the enzyme activity of the breis, it is as a result of a physical or chemical change in the organism being investigated. These changes may be clarified by other properties which the organism exhibits.

The comparatively large (as much as 75%) decrease and subsequent increase in the amount of phosphate release which occurs before first cleavage is difficult to account for on the basis of changes in quantity of enzyme. Several authors have reported cytoplasmic movements after fertilization (see Fankhauser, '48), which may alter the relation of enzyme and substrate to some extent. There is other evidence for changes in the physical state of various eggs: in *Chaetopterus* there are pronounced changes in viscosity before first cleavage (Heilbrun and Wilson, '48); such changes may well have an effect on enzyme activity since they most likely represent an alteration of protein state. As for inactivation of the enzyme as a possible explanation, PPPase is activated by reducing agents (Feinstein and Volk, '49) which apparently do change their state rapidly during development in some cases. For example, in the sea-urchin egg before first cleavage the amount of reduced glutathione decreases 75% and returns to the previous level all within an hour (Rapkine, '31). The possible importance of such physical and chemical changes as those discussed for in vivo conditions is difficult to investigate experimentally since they are usually altered by the experiment itself.

The difference in PPPase activity of individual batches of eggs during early development could be interpreted to mean that the enzyme function is not critical at that time. However, the developing embryo is known to have a flexibility which must have a basis in its metabolism. It is suggested that when

one system fails to function adequately for some reason, the PPPase system may be utilized much as the anaerobic system may substitute for the aerobic one in sugar metabolism. That the deficiency, if any, causing PPPase to function is often inherent in the egg cytoplasm is indicated by the fact that eggs from the same female, whether normal, haploid or hybrid, usually have a similar activity in the earlier stages. The fact that the changes in PPPase activity are not fluctuations but trends of from 10 to 20 hours' duration, which return to a former level, would be in keeping with this hypothesis. At gastrulation, and at neuralation, two processes which are assumed to require energy, the activity is never low.

In the haploid and normal embryos, where gastrulation is successfully concluded, PPPase has in vitro an increased activity before and during gastrulation, whereas in the hybrid, which only begins to gastrulate, the activity is higher only just before gastrulation. Furthermore, the dorsal lip region, which is the most active during gastrulation, has a lower activity in the hybrid than in the normal egg. Can what we know about the metabolism of the amphibians at this stage furnish an interpretation of these results? A distinct possibility is that the phosphate released by the enzyme is directly or indirectly turned into the inorganic phosphate pool to be used in the glycolytic cycle. A careful examination of Gregg's ('48) data on whole eggs reveals that an appreciable amount of glycogen is not utilized until stage 11 to 12, whereas PPPase activity in vitro seems to reach a peak sometime between stage 9 and 10. In the dorsal and ventral lip, however, glycogen decreases between stage 10 and 12 (Jaeger, '45) and PPPase activity is high in both these regions. Correlated with this is a relatively large inorganic phosphate pool at stage 10 (Kutsky, '50). If the apparent lag between highest PPPase activity and glycogen utilization is real, it may be because glycogen utilization is deferred until enough inorganic phosphate has been released for the glycolytic cycle to function; or if the bond is a high energy one, the energy may first be

transferred or used and the inorganic phosphate then released to the glycolytic cycle (Barth and Barth, '51). On the other hand, the enzyme may simply be in such a state that it can more easily be stimulated to react when needed.

In the hybrid the region above the dorsal lip has a lower PPPase activity than it does in the normal gastrula and, correspondingly, less glycogen is utilized in this region (Jaeger, '45). The whole vegetal-animal gradient of PPPase activity seems to be shifted in the hybrid so that the low point is farther toward the vegetal pole. It is of interest to note in this connection that the dorsal lip also seems to be nearer the vegetal pole in the hybrid than in the normal embryo (Moore, '46). This shift may be correlated with the higher enzyme activity in the yolky region rather than with the shift in gradient.

According to recent research of Barth and Jaeger ('50a, b and c, '51), the enzyme PPPase is part of a transfer system; on addition of ATP to a brei containing phosphoprotein, PPPase and ATPase, less inorganic phosphate is found than in the absence of ATP, indicating that the phosphate released has been transferred to another compound by transphosphorylation. There is more ATP and ATPase available for such a process in the dorsal lip than in the ventral ectoderm (Fujii, Utida, Ohnishi and Yanagisawa, '51). The organism may well avail itself of the fact that phosphate can either be released as inorganic phosphate or transferred to another compound.

After the neural tube stage PPPase activity in vitro decreases; until stage 22 this is a function of loss of substrate rather than enzyme (Barth and Barth, '51) although by stage 25 there is also little enzyme (Barth and Jaeger, '50). No appreciable decrease in yolk platelets occurs until after hatching (Bragg, '39) but PPPase could still be active in vivo since it removes phosphate without previous hydrolysis of the protein molecule (Feinstein and Volk, '49). Although no difference in PPPase activity was found between normal and haploid embryos, at stages after hatching this may be the effect

of substrate since haploids have more yolk at these stages (Porter, '39).

Although an investigation of the effect of nuclear factors on enzyme activity during development was not the primary purpose of this study, it is of interest that PPPase activity, at least in vitro, can be maintained at a normal level even though only half the usual amount of genetic material is present. In the hybrid both the amount and distribution of PPPase activity are altered. Moore ('47, '48) suggested that failure of the hybrid to gastrulate would be explained by competition between genes of the two parent species, or their analogs, for substrate so that less product is released. Although in the present study less phosphate is released in the hybrid than in the normal embryo, the experiments were not designed to reveal the mechanism.

SUMMARY

1. Phosphoprotein phosphatase activity during normal, haploid and hybrid amphibian development was determined by measuring the inorganic phosphate released by breis. The phosphate was measured after 10 minutes' incubation of the brei at a temperature of 26°C. and a pH of 3.5, conditions which were found to be nearly optimum for PPPase activity. There is an average activity under these conditions of 4 to 5 μg P per egg.

2. In normal development the PPPase activity decreases slightly after fertilization and shows a pronounced drop and rise in activity before first cleavage and again between the third and 5th cleavage. During later cleavages the activity shows no definite pattern, but just before and during gastrulation the activity is high; after closure of the neural tube the activity declines.

3. Eggs from the same female, whether haploid, hybrid or normal, generally have a similar PPPase activity up to gastrulation.

4. Differences in PPPase activity between haploid and normal embryos, if any, were too small to be detected in the number of experiments performed.

5. The hybrids have an increase in activity of shorter duration than do the normal or haploid embryos.

6. Determination of the PPPase activity per dry weight unit in 6 regions of the early gastrula reveal a lower activity in the region above the dorsal lip in the hybrid than in the normal embryo.

7. The PPPase activity of the normal gastrula regions was calculated per unit of yolk, dipeptidase activity, total nitrogen and per cell.

8. The possible role of PPPase in development was discussed.

ACKNOWLEDGMENTS

I wish to express my appreciation to Dr. L. G. Barth for his guidance and criticism throughout the course of this work.

I wish also the thank Dr. L. C. Sze for valuable discussions and technical advice, Dr. L. J. Barth and Jerome J. Freed for helpful comments and suggestions, Dr. Roberts Rugh for placing at my disposal x-ray equipment and technical assistance, and last, the Statistical Bureau of Columbia University for advice.

LITERATURE CITED

ATLAS, M. 1938 The rate of oxygen consumption of frogs during embryonic development and growth. Physiol. Zool., *11:* 278–291.

BARTH, L. G. 1946 Studies on the metabolism of development. The metabolic nature of the block to gastrulation in hybrid eggs. J. Exp. Zool., *103:* 463–486.

BARTH, L. G., AND L. JAEGER 1947 Phosphorylation in the frog's egg. Physiol. Zool., *20:* 133–146.

———— 1950a The role of adenosine-tri-phosphate in phosphate transfer from yolk to other proteins in the developing frog egg. I. General properties of the transfer system as a whole. J. Cell. and Comp. Physiol., *35:* 413–436.

———— 1950b The role of adenosine-tri-phosphate in phosphate transfer from yolk to other proteins in the developing frog egg. II. Separation of the system into component enzymes, phosphate donor and phosphate acceptor. Ibid., *35:* 437–460.

BARTH, L. G., AND L. J. BARTH 1950c Further studies of phosphate transfer in the frog egg. Anat. Rec., *108:* 587.

———— 1951 The relation of adenosine triphosphate to yolk utilization in the frog's egg. J. Exp. Zool., *116:* 99–122.

BARTH, L. G., AND L. C. SZE 1951 A convenient micropipette. The Rev. Sci. Instr., *22:* 978–980.

———— 1951 Regional chemical differences in the gastrula of *Rana pipiens* (in press).

———— 1952 The diversity of the gastrula cells (to be published).

BRAGG, A. N. 1939 Observations upon amphibian deutoplasm and its relation to embryonic and early larval development. Biol. Bull., *77:* 268–284.

BRIGGS, R., E. U. GREEN AND T. J. KING 1951 An investigation of the capacity for cleavage and differentiation in *Rana pipiens* eggs lacking "functional" chromosomes. J. Exp. Zool., *116:* 455–500.

FANKHAUSER, G. 1948 The organization of the amphibian egg during fertilization and cleavage. Ann. N. Y. Acad. Sci., *49:* 684–708.

FEINSTEIN, R. N., AND M. E. VOLK 1949 Phosphoprotein phosphatase in mammalian tissues. J. Biol. Chem., *177:* 339–346.

FISKE, C. H., AND Y. SUBBAROW 1925 The colorimetric determination of phosphorus. J. Biol. Chem., *66:* 375–400.

FUJII, T., S. UTIDA, T. OHNISHI AND T. YANAGISAWA 1951 The apyrase activity and adenosinetriphosphate content of the organizer region of Bufo vulgaris formosus. Ann. Zool. Jap., *24:* 115–119.

GREGG, J. R. 1948 Carbohydrate metabolism of normal and of hybrid amphibian embryos. J. Exp. Zool., *109:* 119–133.

GREGG, J. R., AND S. LØVTRUP 1950 Biochemical gradients in the Axolotl gastrula. Comp. rend. Lab. Carlsberg Ser. chim., *27:* 307–324.

HARRIS, D. L. 1946 Phosphoprotein phosphatase, a new enzyme from the frog egg. J. Biol. Chem., *165:* 541–550.

HEILBRUNN, L. V., AND W. L. WILSON 1948 Protoplasmic viscosity changes during mitosis in the egg of Chaetopterus. Biol. Bull., *95:* 57–68.

JAEGER, L. 1945 Glycogen utilization by the amphibian gastrula in relation to invagination and induction. J. Cell. and Comp. Physiol., *25:* 97–120.

KUTSKY, P. B 1950 Phosphate metabolism in the early development of *Rana pipiens.* J. Exp. Zool., *115:* 429–460.

MOORE, J. A. 1946 Studies on the development of frog hybrids. I. Embryonic development in the cross *Rana pipiens* ♀ × *Rana sylvatica* ♂. J. Exp. Zool., *101:* 173–220.

———— 1947 Studies in the development of frog hybrids. II. Competence of the gastrula ectoderm of *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrids. J. Exp. Zool., *105:* 349–370.

———— 1948 Studies in the development of frog hybrids. III. Inductive ability of the dorsal lip region of *Rana pipiens* ♀ × *Rana sylvatica* ♂ hybrids. J. Exp. Zool., *108:* 127–154.

———— 1949 Geographic variation of adaptive characters in *Rana pipiens* Schreber. Evolution, *III:* 1–24.

NEEDHAM, J. 1931 Chemical Embryology. Cambridge University Press, Cambridge. Pp. 331–354.

PORTER, K. R. 1939 Androgenetic development of the egg of *Rana pipiens*. Biol. Bull., *77:* 233–267.

RAPKINE, L. 1931 Sur les processus chimique au cours de la division cellulaire. Ann. Physiol., *7:* 382–418.

RUGH, R. 1939 Developmental effects resulting from exposure to x-rays. I. Effect on the embryo of irradiation of frog sperm. Proc. Am. Phil. Soc., *81:* 447–471.

RUGH, R., AND F. EXNER 1940 Developmental effects resulting from exposure to x-rays. II. Development of leopard frog eggs activated by bullfrog sperm. Proc. Am. Phil. Soc., *83:* 607–619.

SHUMWAY, W. 1940 Stages in the normal development of *Rana pipiens*. I. External form. Anat. Rec., *78:* 139–147.

SPIEGELMAN, S., AND H. B. STEINBACH 1945 Substrate-enzyme orientation during embryonic development. Biol. Bull., *88:* 254–268.

SZE, L. C. 1952 Respiration of the parts of the *Rana pipiens* gastrula (in press).

TING, HAN-PO 1950 An aceto-carmine squash technic for amphibian chromosomes. Stain Tech., *25:* 127–128.

COMMENTS AND COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

INFLUENCE OF pH ON THE GROWTH OF TETRAHYMENA IN SYNTHETIC MEDIUM

JOHN V. SLATER[1]

Department of Zoology, University of Michigan, Ann Arbor

ONE FIGURE

The pH optimum for the growth of *Tetrahymena geleii* E. in peptone medium was established by Elliott in 1933 but the most effective pH for the growth of this ciliate in synthetic medium has not been demonstrated. It was shown by Slater ('51) that the medium usually used for the growth of strain E will not hold its initial pH especially when large amounts of glucose are present. The use of piperazine dihydrochloride and glycylglycine, as reported by Smith and Smith ('49) for the production of a non-toxic buffer system might be applicable for *Tetrahymena*.

[1] Postdoctoral Fellow in the Biological Sciences, United States Atomic Energy Commission.

TABLE 1

Effect of initial pH on peak population production of Tetrahymena geleii E. *in synthetic media*

PLUS MINERALS				MINUS MINERALS			
pH Init.	Final	Peak O.D.[1]	Popul. Count	*pH* Init.	Final	Peak O.D.	Popul. Count
5.6	7.1	132	98,000 ± 9,200	5.6	6.6	119	76,000 ± 9,000
6.4	7.2	134	80,000 ± 7,800	6.6	7.1	134	82,000 ± 11,000
7.6	7.6	140	80,000 ± 5,400	7.6	7.1	125	69,000 ± 7,400
8.5	7.5	32	36,000 ± 1,040	8.5	7.9	21	9,900 ± 860

[1] Optical density.

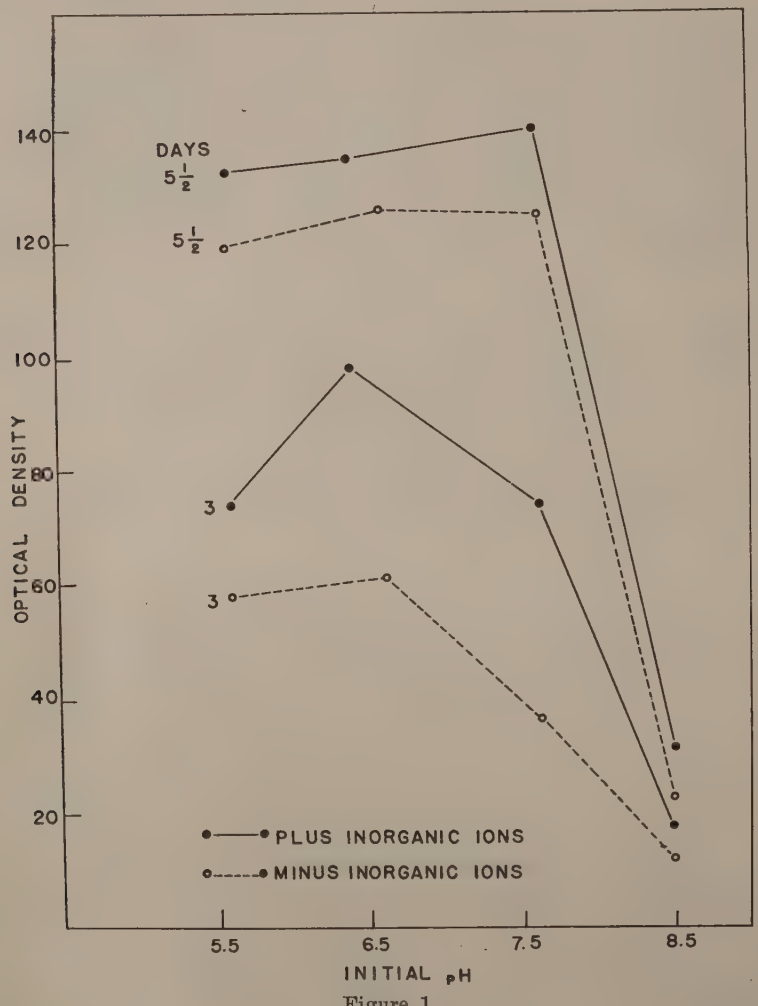


Figure 1

The composition of the medium and the procedures used were modified from Elliott ('49, '50) as previously reported by Slater ('52). Protogen [2] was used instead of α-lipoic acid, and magnesium sulfate to the extent of 10 mcg/ml was present in all instances (Slater, in press). The influence of the usual cation spectrum used was tested for any effect on the pH optimum for growth. None of the other cations were present in the media listed as "minus minerals" except for potassium as the phosphate and calcium as the pantothenate with the exception that the usual contaminations carried in with the analytic reagents were present.

In the presence of the cations, initial pH values from 5.6–7.6 did not effect the peak population production. At pH 6.5 the cations had no effect, but at pH 5.6 or 7.6 somewhat better growth was obtained when the salts were present. It is seen from table 1 that the ciliate was able to regulate its environmental hydrogen ion concentration to a final value of 7.1–7.6 regardless of the initial value when all of the cations were present. Acid production probably lowers the pH when cultures are started at high values, and nitrogenous excretory products raise the pH when cultures are incubated in media started at low pH values. After three days' incubation, cultures in media with the cations present were found to grow best at pH 6.4 (fig. 1), but after 5½ days about the same amount of growth was obtained in media started with an initial pH ranging from 5.5–7.5. The fastest growth rate, however, was obtained at pH 6.4.

The synthetic medium used for the growth of strain E does not yet produce tetrahymenal protoplasm as rapidly as does peptone. Work in this laboratory now in progress by Elliott and Wu (unpublished data) may result in a much improved synthetic environment for this animal.

LITERATURE CITED

Elliott, A. M. 1933 Biol. Bull., *65:* 45.
———— 1949 Physiol. Zool., *22:* 338.
———— 1950 Ibid., *23:* 85.

[2] The aid of E. L. R. Stokstad of the Lederle Laboratories Division, American Cyanamid Company, in supplying the protogen is gratefully acknowledged.

ELLIOTT, A. M., AND C. WU Unpublished data.
SLATER, J. V. 1951 Growth in Tetrahymena in relation to carbohydrate metabolism. Thesis, Univ. of Mich.
———— 1952 Science, *115:* 376.
———— Physiol. Zool. (in press).
SMITH, M. E., AND L. B. SMITH 1949 Biol. Bull., *96:* 233.

CONCENTRATION OF HEMOGLOBIN IN THE BLOOD OF DEEP SEA FISHES

L. VAN DAM AND P. F. SCHOLANDER
Woods Hole Oceanographic Institution, Woods Hole, Massachusetts
(Contribution number 633 from the Woods Hole Oceanographic Institution)

It has long been known that deep sea fishes can secrete gas into their swimbladders against considerable pressures. Richard (1895) and Schloesing and Richard (1896) analyzed the swimbladder gas from deep sea eels, *Simenchelys parasiticus* and *Synaphobranchus pinnatus*, taken at 1674 and 1385 meters' depth respectively, and in a recent study fishes with swimbladders were found to be common down to a depth of at least 1000 meters, or at 100 atmospheres' pressure (Scholander and van Dam, '53). In agreement with the data of Schloesing and Richard we found also that at a pressure of about 100 atmospheres some 90% of the swimbladder gas consists of oxygen.

An important function of the gas secretion is to maintain the fish at a neutral buoyancy at any depth. In order to compensate for a given buoyancy decrease a molar amount of gas must be secreted which is proportional to the hydrostatic pressure at which the buoyancy adjustment must be accomplished. Thus, at 100 atmospheres' depth 100 times more gas must be secreted than at the surface in order to effect the same increase in swimbladder volume. This consideration led us to look for conditions which might possibly speed up or facilitate the gas secretion in deep sea fishes. Such a condition, if the oxygen secreted into the swimbladder

is transported by the hemoglobin, could be an increased hemoglobin content.

Since the iron content of the blood may be assumed to reflect very nearly its hemoglobin content and hence its oxygen combining capacity, we have measured the iron content of the blood in 11 species of deep sea fishes in order to determine if these fish have developed a higher oxygen capacity of the blood to facilitate oxygen secretion.

MATERIAL AND METHODS

The fishes were caught by trawling during two cruises off the New England coast in July, 1952, as previously described (Scholander and van Dam, '53). The blood was drawn by

TABLE 1

Iron content in the blood of deep sea fishes

SPECIES	NUMBER OF INDIVIDUALS	DEPTH IN METERS	MG FE PER 100 CM^3 BLOOD		
			Low	High	Average
Cottunculus thomsonii	3	750–915	10.4	14.1	11.9
Coryphaenoides rupestris	3	390–865	9.2	16.9	11.9
Macrourus berglax	3	860–945	12.8	18.3	16.5
Merluccius bilinearis	1	930	24.0	24.0	24.0
Notacanthus phasganorus	1	610	18.0	18.0	18.0
Sebastes marinus	2	500	24.7	25.1	24.9
Simenchelys parasiticus	3	640–915	20.0	23.7	22.0
Synaphobranchus pinnatus	2	710–865	14.5	29.1	21.8
Urophycis chesteri	2	610–865	16.6	21.7	19.2
Urophycis chuss	2	125	20.4	21.3	20.9
Urophycis tenuis	6	610–640	20.4	26.0	24.0

hypodermic syringe from the heart, which was usually still pulsating. A larger than normal spread in our data may have been brought about by the presence of a severe gas embolism in the blood of some of the fishes, disturbing the normal plasma to cell ratio. The samples (1–2 ml) were stored in small stoppered vials for analysis on shore. No anti-coagulant was used.

The coagulated and more or less rotted sample was transferred quantitatively into a glass homogenizer, and 1 cm^3 of the homogenate was digested by wet ashing in a mixture of

concentrated sulfuric and nitric acid. The concentration of iron in the clear and colorless digest was determined in a Klett-Summerson photo electric colorimeter, essentially following the procedure of Wong ('28).

RESULTS

Our analytical data are shown in table 1. The mean value of the iron content in the blood of 11 species of deep sea fishes is 19.6 mg %. Hall and Gray ('29) give iron values from 15 species of shallow water marine fishes. Their mean value is 26.7 mg %. If we disregard their data on fishes of the mackerel family with an exceptionally high iron content, their average is 22.3 mg %. There is, therefore, no indication that deep sea fishes have higher hemoglobin concentration in their blood than shallow water species.

SUMMARY

The iron content of the blood has been determined in 11 species of deep sea fish, caught at depths of 125 to 945 meters. The values fall well inside the range of those found in shallow water fishes. There is no indication, therefore, that deep sea fishes have developed a high oxygen capacity of the blood as a possible means of facilitating the secretion of oxygen against high pressures.

LITERATURE CITED

Hall, F. G., and I. E. Gray 1929 The hemoglobin concentration of the blood of marine fishes. J. Biol. Chem., *81:* 589–594.

Richard, J. 1895 Sur les gaz de la vessie natatoire des poissons. C. R. Acad. Sci. Paris, *120:* 745–747.

Schloesing, T. and J. Richard 1896 Recherche de l'argon dans les gaz de la vessie natatoire des poissons et des physalies. C. R. Acad. Sci. Paris, *122:* 615–617.

Scholander, P. F., and L. van Dam 1953 Composition of the swimbladder gas in deep sea fishes. Biol. Bull., *104:* 75–86.

Wong, S. Y. 1928 Colorimetric determination of iron and hemoglobin in blood. II. J. Biol. Chem., *77:* 409–412.

NOTICE TO CONTRIBUTORS

THE JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY, appearing bimonthly, is intended as a medium for the publication of papers which embody the results of original research of a quantitative or analytical nature in general and comparative physiology, including both their physical and chemical aspects. Short preliminary notices are not desired and papers will not be accepted for simultaneous publication or which have been previously published elsewhere. While not specifically excluding any particular branch of physiology, contributors should recognize that excellent journals already exist for publication in the field of experimental and physiological zoology, dealing particularly with genetics, growth, behavior, developmental mechanics, sex determination, and hormonal interrelationships, and also for pure mammalian functional physiology and the physical chemistry of non-living systems. Preference will be given to analyses of fundamental physiological phenomena whether the material is vertebrate or invertebrate, plant or animal. Since the journal is restricted, it is not possible to publish more than a limited number of papers which must be short and concise.

It is recognized that prompt publication is essential, and the aim will be to issue papers within three months of acceptance.

Manuscripts and drawings should be sent to the Managing Editor, DETLEV W. BRONK, Office of the President, The Johns Hopkins University, Baltimore 18, Maryland.

The paper must be accompanied by an author's abstract not to exceed 225 words in length, which will appear on the advance abstract cards of the Bibliographic Service of The Wistar Institute in advance of complete publication. Nothing can be done with the manuscript until the abstract is received.

Manuscripts should be typewritten in double spacing on one side of paper $8\frac{1}{2} \times 11$ inches, and should be packed flat — not rolled or folded. The original, not carbon, copy should be sent. The original drawings, not photographs of drawings, should accompany the manuscript. Authors should indicate on the manuscript the approximate position of text figures.

Manuscripts and drawings should be submitted in complete and finished form with the author's complete address. All drawings should be marked with the author's name. The Wistar Institute reserves the privilege of returning to the author for revision approved manuscript and illustrations which are not in proper finished form for the printer. When the amount of tabular and illustrative material is judged to be excessive, or unusually expensive, authors may be requested to pay the excess cost.

The tables, quotations (extracts of over five lines), and all other subsidiary matter usually set in type smaller than the text, should be typewritten on separate sheets and placed with the text in correct sequence. Footnotes should not be in with the text (reference numbers only), but typewritten continuously on separate sheets, and numbered consecutively. Explanations of figures should be treated in the same manner, and, like footnotes, should be put at the end of the text copy. A condensed title for running page headlines, not to exceed thirty-five letters and spaces, should be given.

Figures should be drawn for reproduction as line or halftone engravings, unless the author is prepared to defray the additional cost of a more expensive form of illustration. All colored plates are printed separately and cost extra. In grouping the drawings it should be borne in mind that, after the reduction has been made, text figures are not to exceed the dimensions of the printed matter on the page, $4\frac{1}{2} \times 6\frac{3}{4}$ inches. Single plates may be $5 \times 7\frac{1}{2}$ inches, or less, and double plates (folded in the middle), $11\frac{1}{2} \times 7\frac{1}{2}$ inches. Avoid placing figures across the fold, if possible.

Figures should be numbered from 1 up, beginning with the text figures and continuing through the plates. The reduction desired should be clearly indicated on the margin of the drawing.

All drawings intended for photographic reproduction either as line engravings (black-ink pen lines and dots) or halftone plates (wash and brush work) should be made on white or blue-white paper or bristol board — not on cream-white or yellow-tone. Photographs intended for halftone reproduction should be securely mounted with colorless paste — never with glue, which discolors the photograph.

Galley proofs and engraver's proofs of figures are sent to the author. All corrections should be clearly marked thereon.

The journal furnishes the author fifty reprints, with covers, of the paper gratis. Additional copies may be obtained according to rates which will be sent the author as soon as the manuscript has been examined at The Wistar Institute, after acceptance.

THIS NUMBER COMPLETES VOLUME 41

JOURNAL OF

CELLULAR AND COMPARATIVE PHYSIOLOGY

VOL. 41 JUNE 1953 No. 3

CONTENTS

PRESS OF
THE WISTAR INSTITUTE
OF ANATOMY AND BIOLOGY
PHILADELPHIA

Printed in the United States of America